Chromokinesin Xklp1 Contributes to the Regulation of Microtubule Density and Organization during Spindle Assembly

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Xklp1 is a chromosome-associated kinesin required for *Xenopus* early embryonic cell division. Function blocking experiments in *Xenopus* egg extracts suggested that it is required for spindle assembly. We have reinvestigated Xklp1 function(s) by monitoring spindle assembly and microtubule behavior under a range of Xklp1 concentrations in egg extracts. We found that in the absence of Xklp1, bipolar spindles form with a reduced efficiency and display abnormalities associated with an increased microtubule mass. Likewise, centrosomal asters assembled in Xklp1-depleted extract show an increased microtubule mass. Conversely, addition of recombinant Xklp1 to the extract reduces the microtubule mass associated with spindles and asters. Our data suggest that Xklp1 affects microtubule polymerization during M-phase. We propose that these attributes, combined with Xklp1 plus-end directed motility, contribute to the assembly of a functional bipolar spindle.

INTRODUCTION

At each cell division a spindle-shaped apparatus assembles around the chromosomes and separates sister chromatids toward opposite poles. The spindle is made of two antiparallel arrays of microtubules whose plus-ends interact with each other and with the chromosomes and whose minus-ends focus into the two spindle poles. Spindle assembly relies on the global and local control of microtubule dynamics and on the coordinated activities of molecular motors (Karsenti and Vernos, 2001; Wittmann *et al.*, 2001). At the onset of M-phase, the activation of cyclinB-cdk1 triggers a global change of the cytoplasmic state that results in the increase of microtubule dynamics and the disassembly of the interphase microtubule network (Belmont *et al.*, 1990; Verde *et al.*, 1990). Recent findings strongly support an active role for chromosomes in controlling spindle formation through local phosphorylation-dephosphorylation reactions and by generating a RanGTP gradient, which regulate the activity of factors involved in microtubule nucleation, stabilization, and organization (Karsenti and Vernos, 2001). Besides these “à distance” effects, microtubules and chromosomes also establish direct interactions. As the spindle forms, some microtubules are captured and partially stabilized at specialized sites on the chromosomes called kinetochores, eventually forming the kinetochore fibers that segregate the chromosomes during anaphase. Other chromatin-microtubule interactions occur along chromosome arms and involve kinesinlike proteins belonging to the Kinesin-4 and -10 families (Lawrence *et al.*, 2004). Members of these two families, also known as chromokinesins, are nuclear in in-

terphase and localize to the chromosome arms during M-phase. To date, two such kinesins have been identified in *Xenopus*: Xkid a member of the kinesin-10 family (Antonio *et al.*, 2000; Funabiki and Murray, 2000), and Xklp1, a member of the kinesin-4 family (Vernos *et al.*, 1995). Although they share some characteristics like their plus-end-directed motility and their association with mitotic chromatin, these motors perform nonredundant functions.

Depletion experiments in *Xenopus* egg extracts have shown that Xkid is essential for metaphase chromosome alignment but is not required for bipolar spindle assembly (Antonio *et al.*, 2000; Funabiki and Murray, 2000). Xkid seems to be a typical transporter, powering chromosome arms in an antipoleward manner, therefore contributing to the so-called polar wind involved in chromosome alignment (Carpenter, 1991; Heald, 2000). Although Xklp1 was also originally proposed to participate in chromosome alignment, different experimental strategies have led to the idea that its main function is in spindle formation and maybe cytokinesis (Vernos *et al.*, 1995). Depletion of Xklp1 by antisense oligonucleotide injection in *Xenopus* oocytes resulted in the strong inhibition of early embryonic cell divisions with incomplete furrow ingression and aberrant mitotic figures (Vernos *et al.*, 1995). This approach showed that Xklp1 is essential for the early embryonic cell divisions. Subsequently, a more specific role for Xklp1 in spindle assembly was proposed, based on the spindle phenotypes observed upon addition of anti-Xklp1 antibody to *Xenopus* egg extracts: sperm nuclei formed unstable bipolar spindles with reduced microtubule density and chromosome alignment defects (Vernos *et al.*, 1995), and DNA-coated beads formed either compact microtubule bundles or detached from organized half-spindles (Walczak *et al.*, 1998). These phenotypes suggested that Xklp1 participates in spindle pole extension and mediates chromosome-microtubule interactions. We have now reexamined the role of Xklp1 by monitoring spindle assembly and microtubule behavior in *Xenopus* egg extracts containing different concentrations of this chromokinesin.

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MATERIALS AND METHODS

Xklp1 Depletion

CSF-arrested *Xenopus* egg extracts were prepared as previously described (Murray, 1991). Xklp1 immunodepletion was carried out as described (Antonio *et al.*, 2000). Briefly, protein A-coated magnetic beads (Dyna) were incubated with affinity-purified anti-Xklp1 antibodies (Ab03), in phosphate-buffered saline (PBS)-T (0.01% Triton X-100), for 60 min at 4°C. Beads were washed twice with PBS-T and twice with CSF-XB, added to Cytostatic factor-arrested egg extract (CSF egg extract) and incubated for 60 min on ice with occasional mixing. Beads were retrieved with a magnet and the resulting extracts were used for spindle or aster assembly. Depletion efficiency was monitored by Western blot as previously described (Antonio *et al.*, 2000).

Expression and Purification of Full-length Xklp1

The full-length Xklp1 cDNA (Vernos *et al.*, 1995) was inserted into the plasmid pFastBac and sequenced to confirm the absence of mutations. Recombinant virus was produced with the Bac-to-Bac Baculovirus expression system (Life Technology). For expression, 50 ml of Sf9 cells (2×10^6 cells/ml) were infected for 48 h. For purification, cells were washed in ice-cold PBS and resuspended in 2.5 ml of lysis buffer I (50 mM Na-phosphate, 10 mM KCl, 5 mM β -mercaptoethanol, and protease inhibitors; Complete EDTA-free, Roche). The suspension was homogenized with 25 strokes in a B-type douncer and centrifuged in a JA25-50 Beckman rotor at 27,000 rcf ($\sim 15,000$ rpm) for 10 min at 4°C. The resulting pellet was reextracted with 1 ml of lysis buffer II (50 mM Na-phosphate, 900 mM KCl, 5 mM MgCl_2 , 10% glycerol, 0.1% Tween20, 5 mM β -mercaptoethanol, and protease inhibitors; Complete EDTA-free, Roche) for 30 min on ice. The suspension was homogenized with 25 strokes in a B-type douncer and centrifuged in a JA25-50 Beckman rotor at 27,000 rcf ($\sim 15,000$ rpm) for 10 min at 4°C. The two supernatants were combined and mixed in batch with 1 ml of Talon resin (Clontech, Palo Alto, CA) equilibrated with washing buffer (50 mM Na-phosphate, 250 mM KCl, 10% glycerol, 2.5 mM β -mercaptoethanol, and protease inhibitors; Complete EDTA-free, Roche) and mixed at 4°C for 60 min. The resin was extensively washed with washing buffer containing 15 mM imidazole and loaded on a column. The recombinant protein was eluted with 8×0.5 ml of washing buffer containing 200 mM imidazole.

Spindle Assembly and Aster Experiments

Spindle assembly was induced in *Xenopus* egg extract as previously described (Sawin and Mitchison, 1991). Centrosomal asters were assembled by addition of purified human centrosomes (Bornens and Moudjou, 1999) to CSF extracts containing rhodamine or Cy3-labeled tubulin. For add-back experiments, recombinant full-length Xklp1 (1- to 3-fold endogenous concentration) or the equivalent volume of buffer was added to mock- or Xklp1-depleted extracts when the extract was cycled back into M-phase. For centrosome aster formation in the presence of increasing concentrations of Xklp1, purified centrosomes were incubated with different concentrations of Xklp1 or buffer for 5 min on ice before addition to the CSF extract. For immunofluorescence analysis asters or spindles were diluted with 1 ml of dilution buffer (BRB80, 15% glutaraldehyde, 0.5% glycerol, 1% Triton X-100 for asters, and BRB80, 30% glycerol, 1% Triton X-100 for spindles) and pelleted on coverslips at 22,500 rcf for 12 min at 20°C through a 5 ml glycerol cushion (BRB80, 25% glycerol for asters, 40% glycerol for spindles). Coverslips were fixed for 10 min in cold methanol (-20°C), quenched for 10 min in PBS containing 0.1% sodium borohydrate (Sigma, St. Louis, MO) and processed for immunofluorescence.

Antibodies and Western Blot

Ab03 is a polyclonal antibody raised against a fragment of the stalk domain of Xklp1 (Walczak *et al.*, 1998). P2E9 is a monoclonal antibody (mAb) raised against the tail domain of Xklp1 (Vernos *et al.*, 1995). Tubulin was detected with the mAb DM1 (Sigma) or with in-house-produced polyclonal anti-tubulin antibodies. For immunoblotting, equivalent amounts of treated and untreated egg extracts were resolved on an 8% SDS-gel and transferred onto a nitrocellulose membrane. Membranes were incubated with Ab03 or P2E9 and with a secondary IgG-HRP-conjugate (Dianova). Blots were developed using an ECL (Amersham, Indianapolis, IN) chemiluminescence detection system.

Microtubule Pelleting Assay

Microtubule pelleting assays were carried out as described (Buddle *et al.*, 2001). In brief, centrosomal asters or bipolar spindles were diluted in 500 μl of dilution buffer in the absence of glutaraldehyde and layered onto 500 μl of cushion buffer (see above) in a 1.5-ml Eppendorf tube. Samples were centrifuged at 10,000 rpm ($\sim 12,000$ rcf) for 10 min at room temperature. Supernatants were delicately aspirated, and the interface between supernatant and cushion was rinsed with dilution buffer. Microtubule pellets were dissolved in 30 μl of SDS sample buffer and resolved in 8% SDS-PAGE. Tubulin and Xklp1 contents were analyzed by immunoblot. In add-back experiments, recombinant full-length Xklp1 (at endogenous concentration) or an equivalent

volume of buffer was added to mock- or Xklp1-depleted extracts as the extract was cycled back into M-phase. To induce microtubule depolymerization, nocodazole (Sigma) was added to the extracts after cycling the extracts back to mitosis.

Microtubule Dynamics Assay and Data Analysis

Analysis of microtubule dynamics was carried out as described (Tournéize *et al.*, 1997). Ten microliters of frozen CSF-extract, 0.5 μl of Cy3-labeled tubulin (10 mg/ml), 0.3 μl of human centrosomes (1×10^5 centrosomes/ μl), and 0.5 μl of saturated hemoglobin solution (in CFS-XB) were mixed on ice. This mix, 1.8 μl , was then squashed under an 18×18 -mm coverslip and placed under the fluorescence microscope (all glasses were cleaned with ethanol and rinsed in water). Microtubules were imaged using a Zeiss Axiovert 135 microscope (Thornwood, NY) equipped with a $\times 100$ oil-immersion objective and a long-pass rhodamine filter. Images were captured with a CoolSnap CCD (charged-coupled device) camera controlled by Metamorph software (Universal Imaging, West Chester, PA). In a typical experiment, images were taken every 2 s for 5 min. The four parameters defining microtubule dynamics were determined as follows: microtubule growth (or shrinkage) had to persist for at least six consecutive frames (10 s), with a net change in length of at least 0.5 μm . Catastrophe and rescue frequencies were determined by dividing the number of catastrophe or rescue events by the total time microtubules spent growing or shrinking.

The microtubule steady state length (L) was calculated as described in (Verde *et al.*, 1992) using the equation:

$$\langle L \rangle = \frac{V_g \cdot V_s}{(V_s V_{\text{Fcat}} - V_g V_{\text{Fres}})} (\mu\text{m})$$

Immunofluorescence and Quantifications

For indirect immunofluorescence, coverslips were fixed for 10 min in cold (-20°C) methanol. Fixed samples were then either immediately processed for immunostaining or stored until use at 4°C in PBS. Coverslips were rehydrated for 10 min in PBS-T and incubated first with the primary antibody in PBS-T and then with the secondary antibody (Alexa Fluor-568 or -488 [Molecular Probes] labeled anti-mouse and anti-rabbit antibodies) in PBS-T, both for 20 min at room temperature. DNA was stained with 5 $\mu\text{g}/\text{ml}$ Hoechst dye in PBS-T. For quantifications, nonsaturated images of randomly selected spindles and asters were taken using the same camera settings. The total microtubule fluorescence associated with each spindle and aster was then measured using ImageJ (NIH). Measurement of spindle length and width was carried out using Analysis (Soft Imaging System) and ImageJ software. For analysis of total DNA and tubulin content associated with each spindle, confocal slices of constant thickness were taken throughout the spindles on an LSM-510 confocal microscope (Zeiss) equipped with a $\times 63$ oil-immersion objective. The intensities of microtubules or DNA fluorescence were measured on each slice with ImageJ. The total intensities were calculated by addition of the single slice values. Determination of aster average length and average tubulin content was carried out using a custom-made macro running on Matlab software (MathWorks, Natick, MA), as described in Gruss *et al.* (2002). To discriminate between spindle shapes, we calculated the average width of all spindles assembled in the control extract and defined as barrel-like all spindles wider than the average width + 1 SD. The data were subjected to statistical analysis using an unpaired Student's t test (http://www.physics.csbsju.edu/stats/t_test.html).

RESULTS

Bipolar Spindles Form Less Efficiently in Xklp1-depleted Egg Extracts

To explore further the role of Xklp1 during cell division, we first optimized the conditions to deplete Xklp1 from CSF-arrested *Xenopus* egg extracts. We found that incubation of the extract with affinity-purified anti-Xklp1 antibody-coated beads for 60 min resulted in the effective depletion of Xklp1. Western blot analysis indeed showed that $<1\%$ of Xklp1 was still detectable in the depleted extract (Figure 1A).

To date, two different assembly reactions have been described for promoting spindle formation in *Xenopus* egg extract (Sawin and Mitchison, 1991; Heald *et al.*, 1996). In the "CSF spindle assembly" reaction, each haploid sperm nucleus drives the formation of a half-spindle when incubated in CSF-arrested egg extract and, with time, bipolar spindles form through a pathway that has not been clearly characterized (Sawin and Mitchison, 1991). In the second type of reaction called "cycled spindle assembly," sperm nuclei are

Figure 1. CSF spindle assembly in Xklp1-depleted extract results in the formation of abnormal mitotic figures. (A) Western blot showing the typical efficiency for Xklp1 depletion. Mock-depleted (M) Xklp1-depleted (ΔX ; 1 μ l of each) and various amounts of untreated extract (from 0.1 to 1 μ l as indicated) were resolved by SDS-PAGE. The blot was probed with anti-Xklp1 and anti-tubulin antibodies. The tubulin band was used as a loading control. (B) Images of representative mitotic structures that could be observed both in control and Xklp1-depleted extracts and quantified in panel C (DNA is in blue and rhodamine-labeled tubulin in red). Bar, 10 μ m. (C) Proportion of monopolar (left), bipolar (center), and monopolar abnormal (Mono.abn, right) structures observed in mock- and Xklp1-depleted extracts. The data represent the average ($\pm$ SD) from three independent experiments. In Xklp1-depleted extracts, the majority of structures are monopolar abnormal ($72.9 \pm 8.6\%$), and the other structures are either monopolar ($9.4\% \pm 1.5\%$) or bipolar ($17.7 \pm 7.5\%$) spindles. In mock-depleted extract the majority of structures are monopolar spindles ($56.8 \pm 11.2\%$) and bipolar spindles ($27.6 \pm 6.3\%$), with a small proportion of monopolar abnormal ($15.6 \pm 5.7\%$). For each experiment 110–300 mitotic figures were scored for both types of extracts.

added to CSF-arrested egg extract and the mixture is sent into interphase by addition of calcium. During interphase, DNA is replicated and centrosomes are duplicated. The system is then cycled back into M-phase by addition of fresh CSF-extract and each nucleus assembles a bipolar spindle containing two separated centrosomes, replicated chromosomes, and paired kinetochores.

We first performed CSF spindle assembly reactions using mock- and Xklp1-depleted extracts. Incubation of sperm nuclei in CSF-extract for 45 min resulted in the formation of monopolar half-spindles and bipolar spindles in mock-depleted extracts (Figure 1B). Similar structures were also observed in Xklp1-depleted extracts, but the proportion of bipolar spindles was reduced compared with the control (18% in Xklp1-depleted extract vs. 27% in mock-depleted

Figure 2. Cycled spindle assembly in Xklp1-depleted extracts results in a reduced efficiency of bipolar spindle formation. Percentage of the monopolar and bipolar spindles formed in mock- (blue bars) and Xklp1-depleted (red bars) extracts at 30 and 45 min after cycling the extract back into M-phase. The majority of structures formed in the mock-depleted extracts are bipolar spindles: $54.5 \pm 5.9\%$ at 30 min and $64.3 \pm 5.3\%$ at 45 min. By contrast, in Xklp1-depleted extracts the majority of the structures are monopolar spindles: $62.5 \pm 5.3\%$ at 30 min and $51.9 \pm 8.1\%$ at 45 min. The data are the average $\pm$ SD from three independent experiments, for each experiment 120–350 mitotic figures were scored for both extracts.

extract; Figure 1C). Moreover, we observed that in a majority of the half-spindles assembled in Xklp1-depleted extract, chromatin positioning was incorrect. In these half-spindles, the chromosomes were often immersed in the microtubule array instead of stretching at microtubule-splayed plus-ends as in the control (Figure 1B). Therefore, in accordance with previous experiments, we found that in CSF spindle assembly reactions, Xklp1 depletion affects bipolar spindle formation and chromosome positioning.

We then examined cycled spindle assembly in mock- and Xklp1-depleted extracts. Spindles formed 30 and 45 min after sending the extract back into M-phase were centrifuged onto coverslips and analyzed by fluorescence microscopy. The quantitative analysis of three independent experiments showed that after 30 min mock-depleted extracts contained an average of 55% of bipolar spindles, whereas Xklp1-depleted extracts contained mostly monopolar spindles (63%). At a later time point (45 min), the percentage of bipolar spindles in mock-depleted extracts increased to 65%, but remained low in the Xklp1-depleted extract (52%; Figure 2). Thus, in the absence of Xklp1, bipolar spindles formed both with lower efficiency and with slower kinetics than in control extracts.

Altogether these data suggested that Xklp1 plays a role in positioning chromatin at microtubule plus-ends and in efficient bipolar spindle formation.

Spindles Assembled in Xklp1-depleted Extracts Have an Increased Microtubule Mass

Although quantifying bipolar spindles formed in cycled extracts, we noticed that in the absence of Xklp1 several spindles did not show the typical fusiform shape but appeared to be wider, adopting a barrel-like morphology (Figure 3A). To quantify this phenotype, we arbitrarily classified as barrel-like all spindles whose width was larger than the average width + 1 SD. Results from three independent experiments showed that nearly half of the bipolar spindles assembled in Xklp1-depleted extracts had a barrel-like shape, whereas only 16% showed a similar morphology in mock-depleted extracts (Figure 3B). To try to understand what was causing this phenotype, we first quantified the total amount of polymerized tubulin associated with individual spindles formed in the presence or absence of Xklp1. As shown in

Figure 3. Bipolar spindles formed in Xklp1-depleted extracts have a higher density of microtubules. (A) Representative images of a typical fusiform (Normal) and abnormal (Barrel-Like) spindles formed in Xklp1- and in mock-depleted extracts in the cycled spindle assembly reaction. DNA is in blue and rhodamine-labeled tubulin in red. Bar, 10 μ m. (B) Quantification of normal (white bars) versus barrel-like (black bars) spindles in control (Mock) and Xklp1-depleted (Δ Xklp1) extracts 45 min after cycling the extracts back into mitosis. The graphs show the averages of three independent experiments ($\pm$ SD). In Xklp1-depleted extracts 56.3 $\pm$ 8.1% of the bipolar spindles displayed a barrel-like shape, whereas in control extracts only 15.7 $\pm$ 3.1% had this phenotype. (C) Quantification of the intensity of tubulin fluorescence associated with bipolar spindles assembled under the different conditions. The proportion of spindles in each empirically defined category is shown. The distribution of spindle intensities is shifted to higher values in Xklp1-depleted extracts (blue bars) than those assembled in control extracts (red bars). The data shown are from one representative experiment. Similar results were obtained in three independent experiments. Unpaired Student's *t* test showed that the difference is statistically significant ($p < 0.001$, $t = 5.61$). The number of spindles analyzed in the experiment shown: Mock, $n = 49$; Δ Xklp1, $n = 50$). (D) Average lengths (left) and widths (right) of the bipolar spindles assembled under the two experimental conditions. Spindles had comparable lengths in the two conditions (the small difference is not statistically significant, unpaired *t* test, $p < 0.12$, $t = 1.59$), whereas spindles assembled in Xklp1-depleted extract were wider, as shown by the shift of the width values to the right (these differences are statistically significant, unpaired *t* test, $p < 0.001$, $t = 5.94$). The data are representative of three independent experiments. The number of spindles analyzed in the experiment shown: Mock, $n = 49$; Δ Xklp1, $n = 50$. (E) Spindles assembled in mock- and Xklp1-depleted extracts were optically sliced and the total intensities of tubulin and

Table 1. Quantification of total DNA and tubulin fluorescence intensities in bipolar spindles assembled in mock- and Xklp1-depleted extracts

	Tubulin (a.u.)	DNA (a.u.)
Δ Xklp1	513.9 $\pm$ 204.7	48.3 $\pm$ 24.7
Mock	249.8 $\pm$ 106.5	32.9 $\pm$ 15.1

Quantification of tubulin (left) and DNA (right) fluorescence intensities associated with bipolar spindles assembled in mock- and Xklp1-depleted (Δ Xklp1) extracts. Spindle assembled in Xklp1-depleted extracts had on average higher tubulin fluorescence intensities values than those assembled in control extracts. This difference is statistically significant (unpaired *t* test, $p < 0.001$, $t = 4.73$). Spindles assembled in any of the two extract conditions contained similar amounts of DNA ($p < 0.047$, $t = 2.07$). Values are in means $\pm$ SD. a.u., arbitrary units.

Figure 3C, the distribution of tubulin fluorescence intensities of spindles formed in Xklp1-depleted extract was shifted to higher values than that of spindles assembled in mock-depleted extracts. This indicated that an increase in microtubule mass could be at the origin of the barrel-like spindle phenotype. To determine whether this increase was due to changes in microtubule length and/or number, we measured the length and width of bipolar spindles assembled in the different extracts. We found a similar distribution of spindle lengths for the two types of extracts. However, the distribution of widths of spindles assembled in Xklp1-depleted extract was shifted toward higher values than that of spindles assembled in mock-depleted extracts (Figure 3D). These results (total tubulin fluorescence intensity, spindle length and width) were always consistent in three independent experiments. Finally, to rule out that the increase in microtubule mass of spindles assembled in Xklp1-depleted extract could be related to a higher content of chromatin due, for example, to the clustering of several sperm nuclei, we quantified the fluorescence intensities of both tubulin and DNA in individual spindles formed in the different extracts (Figure 3E). Most spindles assembled in mock- and Xklp1-depleted extracts had similar DNA fluorescence intensity values, although a few contained more chromatin, probably due to sperm nuclei clustering (top part of Figure 3D). However, for similar DNA fluorescence levels, the tubulin fluorescence intensity of spindles assembled in Xklp1-depleted extracts was on average 50% higher than those assembled in mock-depleted extracts (Figure 3E, Table 1). This analysis confirmed that spindles assembled in Xklp1-depleted extracts had a higher microtubule content than those assembled in control extracts.

To rule out that other important protein(s) could be codepleted with Xklp1 during immunoprecipitation, we checked whether adding purified recombinant Xklp1 to Xklp1-depleted extracts could fully rescue the depletion phenotype. We estimated Xklp1 endogenous concentrations to be in the range of 0.4–0.9 μ M in CSF-arrested egg extracts (unpublished data) and added recombinant full-length Xklp1 in the

DNA were measured. Although there are some fused sperm nuclei in the Xklp1-depleted extract (white crosses at the top part of the graph), most spindles formed in this extract have similar DNA contents but higher tubulin content than spindles formed in mock-depleted extract (Δ).

Figure 4. Addition of recombinant Xklp1 to depleted extracts rescues the efficiency of bipolar spindle formation and their fusiform morphology. (A) Top panel, Western blot of control (Mock) or Xklp1-depleted (Δ Xklp1) extracts to which recombinant Xklp1 (Adb) or nocodazole (Noc) were added as indicated. The blot was probed with the anti-Xklp1 antibody and an anti-tubulin antibody. The tubulin band was used as a loading control. (A) Lower panel, Western blot analysis of pellets of spindles assembled in the different extract conditions as indicated. Note that the microtubule pellet from spindles formed in mock-depleted extract is bigger than in Xklp1-depleted extract. In the presence of the microtubule depolymerizing drug nocodazole (Δ Xklp1 +/+ and Mock +/+) the amount of Xklp1 recovered in the pellet is similar to that in the absence of nocodazole, suggesting that most Xklp1 is actually associated with the chromatin. (B) Immunofluorescence images of bipolar spindles formed in Mock-depleted (M) and Xklp1-depleted (Δ X) extracts and after add-back of Xklp1 (Adb; top panel: tubulin is in red, DNA is in blue, and Xklp1 is in green). The bottom panel shows the channel for Xklp1. Bar, 10 μ m. (C) Quantification of the bipolar and monopolar structures (top panel) and of the normal and barrel-like spindles (bottom panel) formed in the three extract conditions. The addition of full-length recombinant Xklp1 (Adb) restores the efficiency of bipolar spindle formation and the morphology of the spindles.

depleted egg extract to restore Xklp1 physiological concentration (Figure 4A). Immunofluorescence analysis showed that the recombinant protein localized to the chromosomes like endogenous Xklp1 (Figure 4B) and that it was able to rescue both the efficiency of bipolar spindle assembly (Figure 4C, top panel) and the fusiform shape of the spindles (Figure 4C, bottom panel).

As a complementary approach to verify that Xklp1 affects microtubule polymerization, spindles assembled in mock- and Xklp1-depleted extracts, supplemented or not with recombinant full-length protein, were pelleted through a glycerol cushion, and the resulting pellets were analyzed by Western blotting. We observed consistently an increase in tubulin in the microtubule pellets obtained from spindles assembled in Xklp1-depleted extracts compared with those assembled in control extracts or in Xklp1-depleted extract containing recombinant protein (Figure 4A). These results again indicated strongly that Xklp1 exerts a negative effect on microtubule polymerization in the M-phase extract.

Altogether the different analysis performed in Xklp1-depleted egg extracts suggested that Xklp1 somehow limits the total mass of microtubules engaged in bipolar spindle formation. In its absence, the efficiency of bipolar spindle formation decreases and spindles have an altered shape that is most probably related to an increase in microtubule number since microtubule length seems unaffected.

Increasing Xklp1 Concentration in the Egg Extract Results in the Reduction of the Spindle Microtubule Mass

We reasoned that if Xklp1 activity limits somehow microtubule polymerization during spindle assembly, increasing its concentration in the egg extract should result in the opposite effect. We therefore increased the concentration of Xklp1 by a factor of 2–3 in the egg extract and examined cycled

spindle formation under these conditions (Figure 5A). We found that bipolar spindles formed as efficiently in the presence of high Xklp1 concentrations as in control extract (unpublished data). However, the quantitative analysis of the tubulin fluorescence intensity of spindles revealed that those assembled in extracts containing more Xklp1 had a lower tubulin content than those assembled in control extracts (Figure 5, B and C). These results were consistent with the previous ones and supported the view that Xklp1 has a negative effect on microtubule polymerization during M-phase.

Xklp1 Associates with Microtubules Nucleated by Centrosomes

To get a further understanding of the role of Xklp1 in limiting microtubule polymerization, we decided to examine microtubule aster formation by purified centrosomes incubated in CSF-egg extract. We first checked whether Xklp1 associated with astral microtubules. Asters formed in mock-depleted extract, Xklp1-depleted extract, or Xklp1-depleted extract containing recombinant Xklp1 were fixed, centrifuged onto coverslips, and processed for immunofluorescence with anti-Xklp1 antibodies. These antibodies decorated faintly astral microtubules along their length in control and Xklp1-depleted extracts containing recombinant Xklp1. As expected, anti-Xklp1 antibodies did not decorate astral microtubules in Xklp1-depleted extracts, indicating that the signal detected in the other conditions was specific (Figure 6A). The association of Xklp1 with astral microtubules was further confirmed by Western blot analysis (Figure 6B). Xklp1 was present in the microtubule pellets obtained from asters formed in mock- and Xklp1-depleted extracts supplemented with recombinant Xklp1 (Figure 6B). Again as expected, no Xklp1 was found in the microtubule pellets from

Figure 5. Spindles formed in the presence of an excess of Xklp1 have reduced microtubule density. (A) Immunoblot showing a control extract (Buffer) and an extract containing recombinant Xklp1 (+2 μ M). The total concentration of Xklp1 in this extract was estimated to be approximately three times the endogenous concentration. The blot was re probed with an anti-tubulin antibody and the tubulin band used as a loading control (bottom panel). (B) Images of representative spindles assembled in control extract (Buffer) or in the presence of excess of Xklp1 (+2 μ M) taken with the same camera settings. Bar, 10 μ m. (C) Quantification of the intensity of tubulin fluorescence in spindles assembled in the two types of extracts. Spindles assembled in the presence of an excess of Xklp1 (+2 μ M, right panel) had on average less tubulin fluorescence than those assembled in control extracts (Buffer, left panel), resulting in a shift of the spindles distribution toward lower tubulin fluorescence values. (+2 μ M, n = 107; Buffer, n = 113).

Xklp1-depleted extracts. Interestingly, we consistently found that the microtubule pellets derived from Xklp1-depleted extract contained more tubulin than those derived from the control (Figure 6, A and B). These data indicated

that both endogenous and recombinant Xklp1 localize to the microtubules nucleated by centrosomes in egg extract.

These results encouraged us to use the aster assay to investigate further the role of Xklp1 in microtubule assembly.

Figure 6. Xklp1 association with microtubules nucleated by centrosomes. (A) Immunofluorescence images of centrosomal asters formed in mock- and Xklp1-depleted egg extracts and after add-back of recombinant protein (Adb). Xklp1 localizes weakly to microtubules in mock and add-back extracts, but no signal is detected in the depleted extract (tubulin is in red and Xklp1 is in green). Bar, 10 μ m. (B) Western blot analysis of pellets of centrosomal asters assembled in the three extract conditions: mock (M), Xklp1-depleted (Δ X) of Xklp1, and with Xklp1 add-back (Adb). Note that the microtubule pellet is bigger in the absence of Xklp1 than in the other conditions.

Xklp1 Reduces Microtubule Polymerization from Centrosomes

Purified centrosomes were incubated in mock- and Xklp1-depleted extracts, fixed, and pelleted onto coverslips (Figure 7). The total tubulin fluorescence intensity associated with individual asters was then measured and the asters were classified into empirically defined categories of fluorescence intensity (Figure 7B). Most asters assembled in mock-depleted extracts (52%) had low tubulin fluorescence intensity values and only 14% belonged to the highest tubulin fluorescence intensity category. By contrast, asters assembled in Xklp1-depleted extracts showed an inverse distribution: the largest number had high tubulin fluorescence intensity values (51%), whereas only 18% corresponded to the lowest tubulin fluorescence intensity category.

We verified the specificity of this effect by performing add-back experiments. Xklp1-depleted extracts were supplemented with either 0.8 or 1.6 μ M of recombinant Xklp1. Centrosomes were added and the resulting asters were analyzed as above (Figure 7A). Addition of recombinant Xklp1 to the depleted extract, at concentrations close to endogenous levels (0.8 μ M), did shift the aster distribution toward the lower tubulin fluorescence intensity categories. This shift was even more pronounced in depleted extract containing higher concentrations of recombinant Xklp1: under these conditions, 80% of the asters belonged to the lowest tubulin fluorescence intensity category (Figure 7B).

The increase in tubulin content of asters formed in Xklp1-depleted extract could be due to an increase in microtubule length and/or to an increase in the number of microtubules per aster. To investigate this issue, we determined the average length of microtubules in asters formed in the different extracts. We found a mild increase for this value in asters

Figure 7. Effect of Xklp1 concentration on the nucleation of microtubules by purified centrosomes incubated in egg extracts. (A) Western blot analysis of the different extracts used for this assay, probed with anti-Xklp1 and anti-tubulin antibodies. M, mock; ΔXklp1, Xklp1-depleted extract; 0.8, Xklp1-depleted after addition of 0.8 μ M recombinant Xklp1; 1.6, Xklp1-depleted after addition of 1.6 μ M recombinant Xklp1. (B) (left) Distribution of asters assembled in the different extract conditions according to their total tubulin fluorescence intensity values. Asters were classified into five categories defined empirically in intervals corresponding to 50 arbitrary units (a.u.) of fluorescence intensity. The percentage of asters corresponding to each category is shown. In Xklp1-depleted extracts, the majority of asters have a high tubulin fluorescence value (>150 a.u.), whereas the majority of asters assembled in mock-depleted extracts have low tubulin fluorescence values (<50 a.u.). Addition of recombinant Xklp1 to the depleted extract at endogenous concentrations shifts the distribution to lower values of tubulin fluorescence. Increasing the concentration of Xklp1 promotes the formation of asters with low tubulin fluorescence values, restoring a profile that is similar to the control. On the right side, representative images of centrosomal asters formed in the different extracts; Mock, $n = 42$; ΔXklp1, $n = 41$; 0.8 μ M, $n = 52$; 1.6 μ M, $n = 56$. Bar, 10 μ m.

assembled in Xklp1-depleted extracts versus mock-depleted extracts (Table 2). Interestingly, there was a direct correlation between the concentration of Xklp1 in the extract and

Table 2. Average microtubule length in asters assembled in the different types of M-phase extract

CSF extract	MT length (μ m)
Mock	8.3 $\pm$ 2.9
ΔXklp1	10.5 $\pm$ 2.9
ΔXklp1 + 0.8 μ M	9.2 $\pm$ 2.7
ΔXklp1 + 1.6 μ M	7.3 $\pm$ 2.7

Purified centrosomes were incubated in mock-depleted extract (Mock), Xklp1-depleted extract (ΔXklp1), or Xklp1-depleted extract containing the indicated concentration of recombinant full-length Xklp1 (ΔXklp1 + 0.8 μ M and ΔXklp1 + 1.6 μ M). An unpaired Student's *t* test was used to ascertain whether the differences in aster size in the different extracts were statistically significant. The difference between ΔXklp1 and mock is statistically significant ($p < 0.021$, $t = 2.40$), whereas the difference between ΔXklp1 and ΔXklp1 + 0.8 μ M is not statistically significant ($p < 0.14$, $t = 1.49$), but the difference between ΔXklp1 and ΔXklp1 + 1.6 μ M is statistically significant ($p < 0.003$, $t = 3.89$). Values are means $\pm$ SD.

the average length of microtubules in asters (ΔXklp1 > ΔXklp1 + 0.8 μ M > Mock > ΔXklp1 + 1.6 μ M). Addition of recombinant Xklp1 to the depleted extract rescued microtubule length. However, the small change in microtubule length could not account for the average 10-fold difference in total tubulin fluorescence intensity between asters assembled in mock- and Xklp1-depleted extracts. These results suggested that asters formed in Xklp1-depleted extracts have more microtubules than those assembled in mock-depleted extracts.

Xklp1 Depletion Has a Mild Influence on Microtubule Rescue and Catastrophe Frequencies

One possible way by which Xklp1 could influence microtubule polymerization is by changing the parameters defining microtubule dynamic instability. We therefore followed individual microtubules nucleated by centrosomes in mock- and Xklp1-depleted M-phase extracts by time-lapse fluorescence microscopy and determined the four parameters defining microtubule dynamic instability in the presence and absence of Xklp1 (Table 3). The velocities of microtubule growth and shrinkage did not change significantly in the presence or absence of Xklp1. However, there was a mild effect on the frequencies of rescues and catastrophes. In Xklp1-depleted extracts: microtubules rescue frequency increased by 62% (1.17 vs. 0.72 events/min) and the catastrophe frequency increased by 20% (2.49 vs. 2.08 events/min; Table 3). The slightly stronger effect on the rescue frequency suggested that microtubules could be longer in Xklp1-depleted extract. Using the measured parameters, we calculated a steady-state microtubule length of 5.092 μ m for asters assembled in mock-depleted extracts and 5.598 μ m for asters assembled in Xklp1-depleted extracts (Verde *et al.*, 1992). This could explain the increase average size of microtubules measured experimentally for asters assembled in Xklp1-depleted extracts versus mock-depleted extracts. However, this could not account for the increase in microtubule mass observed in spindles and asters assembled in Xklp1-depleted extracts. Again this suggested that the primary effect of Xklp1 depletion is an increase in microtubule number.

Table 3. Effects of Xklp1 depletion on microtubule dynamics

	Vg ($\mu\text{m}/\text{min}$)	Vs ($\mu\text{m}/\text{min}$)	Fcat (events/min)	Fres (events/min)
Mock (n = 30)	9.3 $\pm$ 3.2 (32)	26.4 $\pm$ 7.8 (26)	2.08 (33)	0.72 (7)
ΔXklp1 (n = 31)	11.1 $\pm$ 4.9 (25)	25.6 $\pm$ 10.3 (26)	2.49 (40)	1.17 (15)
	$\langle L \rangle$ (μm)			
Mock	5.092			
ΔXklp1	5.598			

Average velocities of microtubule growth (Vg) and shrinkage (Vs) and the catastrophe (Fcat) and rescue (Fres) frequencies in control (Mock) and Xklp1-depleted (ΔXklp1) extracts. n = the number of microtubules observed; the number of observed events are shown in parentheses. The values obtained were used to calculate the steady state length of microtubules ($\langle L \rangle$) are shown in the lower section.

Xklp1 Affects the Early Stages of Centrosomal Microtubule Elongation

We then tried to find an experimental setting that would enable us to examine a potential effect of Xklp1 on the early steps of microtubule nucleation versus a more general effect on microtubule assembly. M-phase extracts were supplemented with buffer, 0.5 or 1 μM recombinant Xklp1, and purified centrosomes. Samples were taken after 1 min to examine the phases of microtubule nucleation and early elongation and after 10 min to examine asters at steady state (Figure 8). At 1 min, asters assembled in the presence of the higher amount of recombinant Xklp1 (1 μM) had a reduced microtubule mass compared with the other conditions (Figure 8A, top panel, and Table 4). This effect was much stronger after 10 min of incubation and was also obvious in samples containing lower concentrations of recombinant Xklp1 (Figure 8A, bottom panel, and Table 4). These data indicated that Xklp1 influences microtubule aster formation from very early on when microtubule nucleation and/or early elongation is the limiting factor. It suggests that Xklp1 has some role in limiting the efficiency of microtubule elongation off the centrosome leading to a decrease in microtubule number and therefore in total microtubule mass.

DISCUSSION

Previous work has showed that injection of antisense oligonucleotide targeting Xklp1 mRNA in *Xenopus* oocytes undermines embryonic cell divisions (Vernos *et al.*, 1995). The major defects observed in the injected oocytes were the lack of cell cleavage and the presence of abnormal mitotic figures. These phenotypes did not provide enough information to decide whether the primary defects occurred during spindle assembly or during furrow ingression and cytokinesis or both. In fact, there is now strong support for an important role of Xklp1 orthologues in central spindle organization during anaphase and cytokinesis (Brust-Mascher *et al.*, 2004; Kurasawa *et al.*, 2004; Kwon *et al.*, 2004; Mazumdar *et al.*, 2004; Zhu and Jiang, 2005). However, further experimental approaches based on inhibitory antibody addition to egg extracts supported strongly the view that Xklp1 has an important role in spindle assembly in *Xenopus* egg extracts containing different concentrations of this protein: from its depletion to up to three times its endogenous levels (by addition of full-length recombinant protein).

The Role of Xklp1 in Pole Extension and Chromosome Positioning

The phenotypes observed previously in egg extracts containing anti-Xklp1 antibodies upon addition of sperm nuclei and chromatin beads suggested that Xklp1 takes part in spindle pole extension and chromosome alignment (Vernos *et al.*, 1995; Walczak *et al.*, 1998). We show here that depletion of Xklp1 from *Xenopus* egg extracts does not completely impair spindle formation. However, in the absence of Xklp1 bipolar spindles need more time to assemble and, at any given time, the percentage of fully formed bipolar spindles is lower than in control extracts. This suggests that although Xklp1 is not a major player in establishing spindle bipolarity, it contributes to its efficiency.

We did not observe major defects in chromosome alignment in spindles formed in Xklp1-depleted cycled extracts. This suggests that the relatively strong chromosome misalignment defects observed previously upon anti-Xklp1 antibody addition to the extract may not have been solely due to the direct inhibition of Xklp1 but could have arisen through some indirect effect due to the presence of the antibody on the surface of the chromosome (Vernos *et al.*, 1995). However, we have now observed that chromosomes are mispositioned in the majority of half-spindles formed in Xklp1-depleted noncycled extracts. This suggests that the balance of forces involved in positioning chromosomes may differ between cycled and noncycled extracts. In fact, sperm chromosomes incubated in CSF extract do not replicate before inducing half-spindle formation. Therefore, in noncycled extracts kinetochores are not paired and may be functionally immature and unable to establish a bipolar attachment. As a result it is possible that in these conditions, chromosomes are more sensitive to the imbalance of forces as suggested by the mispositioning of chromosomes in the absence of Xklp1. Altogether, these data indicate that Xklp1 has indeed some role in moving or maintaining chromosome arms at microtubule plus-ends. Recent studies *in vitro* have indeed shown that Xklp1 is a plus-end directed motor (Bringmann *et al.*, 2004), and it is thus possible that it participates in the generation of forces moving chromosome arms in an antipoleward manner and/or in pole extension as previously proposed. However, our results indicate that Xklp1 does not provide the major forces involved in chromosome positioning in egg extract. Most of these forces are most likely powered by another *Xenopus* chromokinesin, Xkid (Antonio *et al.*, 2000; Funabiki and Murray, 2000).

Figure 8. Effect of increasing Xklp1 concentration on centrosome nucleated asters in egg extract. Microtubule asters were formed by addition of purified centrosomes to CSF-arrested extracts after addition of buffer (blue triangles), 0.5 μM (red dots), or 1 μM (green squares) recombinant Xklp1 and examined after 1 and 10 min. The tubulin fluorescence intensity of individual asters was then plotted against the average microtubule length of the same aster. (A) Top panel, asters formed after a 1-min incubation. Asters are not yet at steady state and their tubulin content is strongly dependent on the microtubule nucleation rate and early elongation from the centrosome. At this time point we observed a linear correlation between the microtubule average length and the total tubulin fluorescence intensity under the three experimental conditions. Note that in the presence of 1 μM recombinant Xklp1 the total tubulin fluorescence intensity is lower than in the other two conditions for similar microtubule lengths (see also Table 4). Bottom panel, asters formed after 10 min of incubation. At this time point asters have reached their steady state length. In this case the total tubulin fluorescence intensity is determined by the microtubule nucleation rate at the centrosome and their dynamic instability properties. Asters nucleated in extract containing 0.5 or 1 μM recombinant Xklp1 have a lower tubulin content than controls (see also Table 4). (B) Western blot probed with anti-Xklp1 and anti-tubulin antibodies of the different extracts used for this assay (Buf, control extract with buffer; 0.5, extract containing 0.5 μM recombinant Xklp1; 1.0, extract containing 1 μM recombinant Xklp1).

Control of Spindle Microtubule Polymer Mass by Xklp1

Centrosomal asters and spindles assembled in cycled extracts containing different concentrations of Xklp1 yield consistent phenotypes, suggesting that Xklp1 regulates negatively microtubule density in M-phase extracts. Changes in the parameters

Table 4. Average microtubule length and total tubulin fluorescence intensities of centrosomal asters assembled in extracts containing increasing Xklp1 concentrations

	Length (μm)	Intensity (a.u.)	Ratio (a.u.)
1 min			
Buffer	3.7 ± 0.6	6288 ± 2413	1715 ± 625
0.5 μM	3.5 ± 0.5	6359 ± 1647	1798 ± 443
1.0 μM	3.2 ± 0.4	4267 ± 1463	1335 ± 432
10 min			
Buffer	7.9 ± 1.2	33378 ± 16495	4093 ± 1607
0.5 μM	8 ± 1.2	20103 ± 9451	2510 ± 1041
1.0 μM	7.9 ± 1.7	19378 ± 9304	2421 ± 934

Asters were analyzed after 1 min (top panel) and 10 min (lower panel) of incubation of centrosomes in extract. The lower ratio of intensity/length calculated for asters assembled in the presence of an excess of Xklp1 indicate that their lower tubulin content is not only due to shorter microtubules but also to their lower number. Values are in means $\pm$ SD. a.u., arbitrary units.

defining microtubule dynamics have a direct influence on the length and mass of polymerized tubulin at steady state. In M-phase, microtubules become short and highly unstable. This is due to a several fold increase in microtubule catastrophe frequency as the systems change from interphase to M-phase (Verde *et al.*, 1992; Karsenti and Vernos, 2001). The major catastrophe factor in the M-phase *Xenopus* egg extract is XKCM1, a Kinesin-13 family member. XKCM1 depletion results in a major increase of microtubule length and this impairs spindle formation in egg extract (Walczak *et al.*, 1996). Xklp1 depletion results in a much milder phenotype. Microtubules are slightly longer but the increase in total microtubule polymer in centrosomal asters and spindles is proportionally much higher, suggesting that the major effect is an increase of microtubule number.

We considered the possibility of a direct negative influence of Xklp1 on the rate of microtubule nucleation in M-phase and tested this idea by comparing the number of microtubule asters induced by RanGTP in mock-depleted and Xklp1-depleted extracts. In our experimental setting we did not see a difference in the ability of RanGTP to induce microtubule aster formation in M-phase egg extract in the absence or presence of Xklp1 (unpublished data). However, we observed a small decrease in total tubulin mass in the early phases of centrosome-directed aster formation in egg extract containing an excess of Xklp1. Because Xklp1 does not localize to the centrosome it is unlikely that it has a role in modulating microtubule nucleation per se. However, our data suggest that it limits microtubule early elongation off the centrosome. The parameters for dynamic instability in Xklp1-depleted extract suggest that microtubules are slightly more dynamic when Xklp1 is absent. Because the rescue frequency increases proportionally more (62%) than the catastrophe frequency (20%), this could explain why at steady state there are more microtubules under conditions of high microtubule nucleation frequency as is the case around mitotic chromosomes or centrosomes.

Recent studies on the kinetic properties of Xklp1 motor domain have shown that it strongly influences microtubule dynamics, slowing down both microtubule growth and shrinkage in asters formed in pure tubulin solution (Bringmann *et al.*, 2004). Motor, at 10 μM , was required to block microtubule growth completely (in the presence of a large excess of tubulin), whereas 1 μM was sufficient to inhibit microtubule depolymerization completely (in the absence of free tubulin). In the

extract, we estimated a concentration for Xklp1 of $\sim 0.4\text{--}0.9\ \mu\text{M}$ in solution. This is in the lower range of the effective concentration producing a clear effect in vitro. Although we do not know the concentration of Xklp1 on the chromosome arms, it seems to be highly enriched there and therefore the in vitro results suggested that its depletion could result in more dramatic spindle phenotypes. However, there might be several reasons to account for this in addition to the concentration factor: 1) some of the properties of the full-length protein may differ from those of the motor domain alone, and 2) several other factors are involved in the regulation of microtubule dynamics in the M-phase egg extract and it is difficult to predict how Xklp1 may cooperate or compete with them for microtubule binding.

Nevertheless, the effect of Xklp1 depletion on microtubule catastrophe and rescue frequencies in the egg extract is in accordance with the measurements made with Xklp1 motor domain in vitro. The decrease of microtubule rescue and catastrophe frequencies by Xklp1 in the extract certainly results in a reduction of microtubule dynamics. Altogether, this could contribute to the stabilization of microtubules around chromosomes, as was previously suggested (Vernos *et al.*, 1995; Walczak *et al.*, 1996) and at the same time it may decrease the overall microtubule number. In this context, the barrel-like shape of spindles assembled in the absence of Xklp1 could arise both from an increase in microtubule number and from the inefficient movement of microtubules toward the spindle poles. Recent studies aimed at understanding the mechanisms that govern spindle length in the *Xenopus* egg extract system have lead the authors to propose the existence of a hypothetical tensile element or matrix attached to the poles and extending toward the spindle equator (Mitchison *et al.*, 2005). Another nonexclusive possibility is that this tensile element may force the spindle to adopt a barrel-like morphology when the forces provided by the chromokinesin are absent and/or the number of microtubules present is larger.

Although we can visualize the mitotic spindle as a finite entity, it is in fact an extremely dynamic assembly of molecules (Karsenti and Vernos, 2001; Wittmann *et al.*, 2001). Several key features are integrated into this self-organizing process: microtubule length, microtubule organization, and spindle volume. Here we have shown that the *Xenopus* kinesinlike protein Xklp1 participates in this process by exerting a control on the number of microtubules in the spindle. Preliminary experiments indicate that this may be an important function because spindles formed in Xklp1-depleted extract do not seem to be fully competent for chromosome segregation (unpublished data). Further experiments will be needed to determine how important this mechanism is for spindle functionality.

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