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Xenopus tropicalis egg extracts provide insight into scaling of the mitotic spindle

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The African clawed frog *Xenopus laevis* has been instrumental to investigations of both development and cell biology, but the utility of this model organism for genetic and proteomic studies is limited by its long generation time and unsequenced pseudotetraploid genome. *Xenopus tropicalis*, which is a small, faster-breeding relative of *X. laevis*, has recently been adopted for research in developmental genetics and functional genomics, and has been chosen for genome sequencing. We show that *X. tropicalis* egg extracts reconstitute the fundamental cell cycle events of nuclear formation and bipolar spindle assembly around exogenously added sperm nuclei. Interestingly, *X. tropicalis* spindles were ~30% shorter than *X. laevis* spindles, and mixing experiments revealed a dynamic, dose-dependent regulation of spindle size by cytoplasmic factors. Measurements of microtubule dynamics revealed that microtubules polymerized slower in *X. tropicalis* extracts compared to *X. laevis*, but that this difference is unlikely to account for differences in spindle size. Thus, in addition to expanding the range of developmental and cell biological experiments, the use of *X. tropicalis* provides novel insight into the complex mechanisms that govern spindle morphogenesis.

Introduction

The African clawed frog *Xenopus laevis* has been instrumental in studies of gene regulation and development (Brown, 2004), and in the past two decades, unfertilized eggs have been used to prepare cell-free extracts that have made important contributions to our understanding of cell cycle regulation (Murray, 1991), DNA replication (Dasso and Newport, 1990), and spindle assembly (Desai et al., 1999). However, *X. laevis* suffers from a long generation time that makes forward genetics prohibitively time consuming, as well as a pseudotetraploid genome that would obscure many phenotypes. The lack of genomic information has limited homology-based searches to existing EST libraries and complicated protein identification by mass spectrometry. *Xenopus tropicalis*, which is a small, faster breeding, diploid relative of *X. laevis*, has recently been adopted for research in developmental genetics and functional genomics, and it is undergoing genome sequencing (Hirsch et al., 2002; Klein et al., 2002). We investigated whether *X. tropicalis* egg extracts could be used for in vitro cell biology experiments and found that they could similarly reconstitute the fundamental cell cycle events of nuclear formation and bipolar spindle assembly around exogenously added sperm nuclei. *X. laevis* antibodies revealed similar staining patterns on *X. tropicalis* spindles and precipitated homologous proteins detected as single isoforms by Western blot, in contrast to the multiple *X. laevis* variants. *X. tropicalis* spindles were significantly smaller than *X. laevis* spindles formed around the same chromosome source. Extract-mixing experiments revealed the presence of cytoplasmic factors that regulate spindle size in a dynamic and dose-dependent fashion. Measurement of individual microtubule dynamics and spindle poleward flux rates did not reveal differences likely to account for the observed changes in spindle size. We propose that microtubule regulatory factors in *X. tropicalis* extracts respond differently to stabilizing agents, such as chromosomes, to generate smaller microtubule structures. Thus, *X. tropicalis* eggs can be used in the same types of assays previously established for *X. laevis*, benefit from more molecular and genetic tools, and be used to investigate interesting cellular scaling phenomena, such as spindle size regulation.

Results and discussion

We set out to evaluate the utility of *X. tropicalis* for in vitro cell biological and biochemical investigations. *X. tropicalis* eggs

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Abbreviations used in this paper: CSF, cytostatic factor; HCG, human chorionic gonadotropin; NuMA, nuclear mitotic apparatus protein; Xnf7, *Xenopus* nuclear factor 7.

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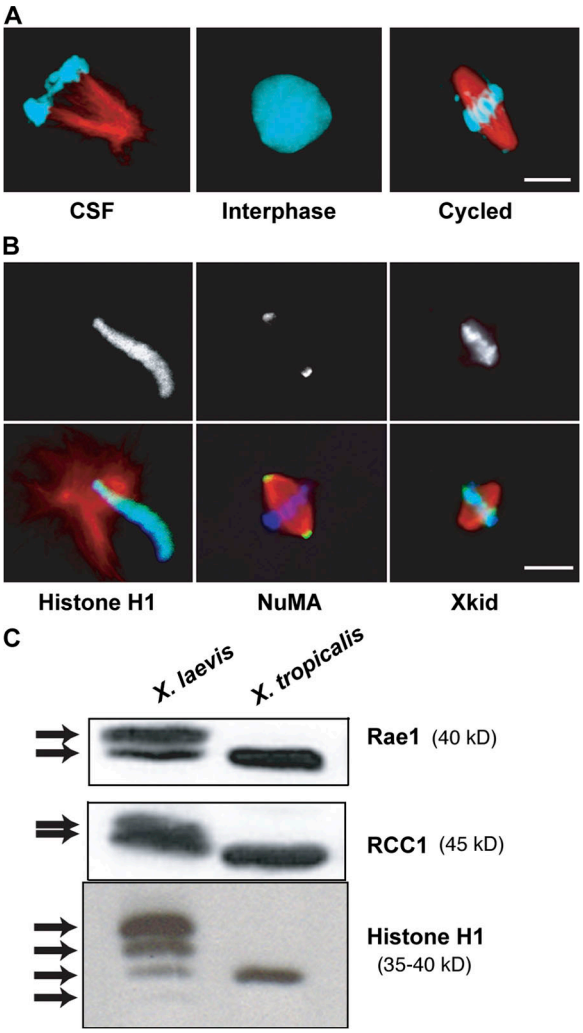

Figure 1. ***X. tropicalis* egg extracts recapitulate major cell cycle events in vitro.** (A) CSF-arrested egg extracts prepared from eggs of *X. tropicalis* and supplemented with *X. laevis* sperm nuclei and X-rhodamine–labeled tubulin formed "half spindles" in metaphase-arrested extracts, interphase nuclei after release from CSF arrest, and bipolar spindles when induced to reenter metaphase. Microtubules are red and DNA is blue. (B) *X. tropicalis* extract reactions were stained with fluorescently labeled antibody (α-H1 and α-NuMA), or by immunofluorescence (α-Xkid) using antibodies raised to the *X. laevis* proteins. Staining patterns recapitulated those in *X. laevis* reactions. In overlays, the stained protein is green. (C) CSF extracts pooled from eggs of multiple *X. laevis* or *X. tropicalis* frogs were blotted for Rae1, RCC1, and histone H1. In *X. laevis* extracts, all proteins gave multiple bands, presumably because of multiple genes for each protein, whereas *X. tropicalis* contained single isoforms. Bars, 10 μm.

(~0.6 mm diam) are approximately one fifth the volume of those of *X. laevis* (1.2 mm diam). To test whether *X. tropicalis* eggs could be used to prepare functional cellular extracts, we collected, dejellied, and crushed unfertilized eggs, which, like those of *X. laevis*, are arrested in metaphase of meiosis II by cytostatic factor (CSF) activity (Murray, 1991). Metaphase-arrested *X. tropicalis* egg extracts assembled spindle structures around exogenously added sperm nuclei, entered interphase, and replicated DNA when released from the arrest, and then cycled back into mitosis (Fig. 1 A). Although yields of extract per frog were 10–20% that of *X. laevis, X. tropicalis* egg extracts effectively recapitulated cell cycle events in vitro.

The utility of *X. tropicalis* extracts would be maximized if reagents generated for *X. laevis* could be applied. Fluorescence microscopy revealed that antibodies against *X. laevis* histone H1 (chromatin component; Maresca et al., 2005a), nuclear mitotic apparatus protein (NuMA; spindle pole component; Merdes et al., 1996), and *Xenopus* kinesin-like DNA-binding protein (Xkid; Funabiki and Murray, 2000) gave identical staining patterns in *X. tropicalis* extracts, compared with *X. laevis* (Fig. 1 B and not depicted). Furthermore, the addition of an inhibitory antibody to Xkid resulted in chromosome congression defects in *X. tropicalis* spindle reactions that were very similar to those observed in *X. laevis* (unpublished data), suggesting that many reagents will be useful in both species. Because *X. laevis* has a pseudotetraploid genome, many genes are present in multiple copies, and without selective pressure, some may be expressed but may not be functional, like one of the Vg1 isoforms (Birsoy et al., 2005). Whether or not the isoforms are functional, there is frequently more than one. *X. laevis* Rae1 (mRNA export factor/spindle regulator; Blower et al., 2005), RCC1 (guanine exchange factor for Ran; Nishitani et al., 1990), and histone H1 were represented by multiple bands on a Western blot, whereas *X. tropicalis* contained a single band for each protein (Fig. 1 C). This suggests that the pseudotetraploid genotype of *X. laevis* contributes to the complexity of the proteome, and the use of *X. tropicalis* could simplify protein analysis.

X. laevis biochemistry is not underpinned by genomic information, making identification of proteins by mass spectrometry difficult. To test whether *X. laevis* proteins could be identified using the *X. tropicalis* database, we immunoprecipitated the microtubule-associated developmental regulator *Xenopus* nuclear factor 7 (Xnf7; Maresca et al., 2005b) from both *X. laevis* and *X. tropicalis* extracts (Fig. 2 A), and then used MALDI mass spectrometry to identify each protein using databases from both species. Xnf7 was identified from both immunoprecipitates using either database (Fig. 2 B), although the number of peptides identified was higher when queried against the database of the same species. Conceptual trypsin digestion of Xnf7 from both species and comparison of the peptides revealed that although the two proteins are highly conserved (Fig. 2 C), only 45% of the peptides have identical masses (not depicted). This analysis suggests that although the *X. tropicalis* database will greatly facilitate the identification of *X. laevis* proteins by mass spectrometry, it will be more efficient to identify homologous *X. tropicalis* proteins.

While validating the use of *X. tropicalis* extracts, we noticed that spindles assembled around *X. laevis* sperm nuclei in *X. tropicalis* extracts were considerably smaller (~30% shorter) than those assembled in *X. laevis* extract (Fig. 3 A). Comparing the fluorescence area of the two types of spindles revealed an approximately threefold difference (unpublished data), indicating a substantially greater microtubule mass in *X. laevis* spindles compared with those of *X. tropicalis*. This prompted us to examine the poorly understood phenomenon of spindle scaling further.

Spindle sizes were extract dependent, suggesting that they may be defined by diffusible cytoplasmic components. To determine whether these factors reflect a balance of activities present

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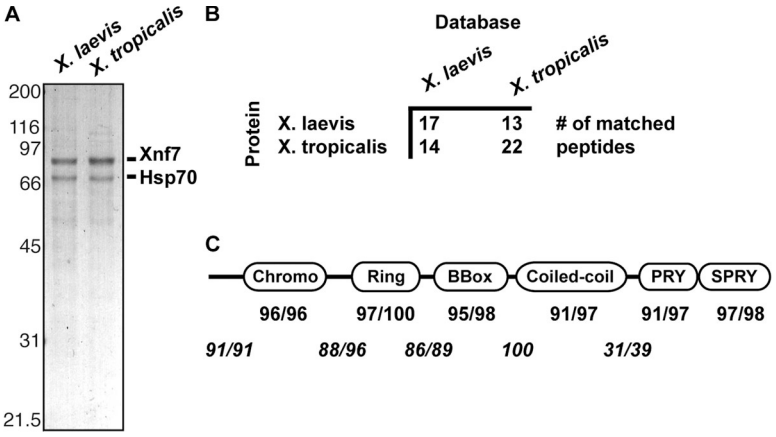

Figure 2. **Identification of *X. tropicalis* and *X. laevis* Xnf7 by mass spectrometry.** (A) Xnf7 was immunoprecipitated from *X. laevis* or *X. tropicalis* extracts and subjected to SDS-PAGE. Excised bands were processed for MALDI-TOF mass spectrometry. (B) Xnf7-derived peptide masses from each band were compared against databases from both *Xenopus* species. The number of peptides that showed a perfect match against each database is shown. (C) Schematic representation of the Xnf7 domain structure showing the number of identical/conserved amino acids in each domain. Domains are in regular font and linker regions are in italics.

in both extracts, or a dominant activity in one of the extracts, we combined extracts in different proportions and examined spindle length in the mixed reactions. We found that spindle length had a direct and linear dependence on the proportion of *X. laevis* to *X. tropicalis* extract (Fig. 3 B), suggesting equilibrium behavior of cytoplasmic regulatory activities. Previous work has also demonstrated a role for chromatin mass in determining spindle size (Nicklas and Gordon, 1985). To investigate this phenomenon, we compared spindle length in *X. laevis*, *X. tropicalis*, and mixed extracts using sperm from *X. tropicalis*, whose diploid genome (1.7×10^9 bp) is ~55% that of *X. laevis* (3×10^9 bp; Hirsch et al., 2002), and found that spindles assembled around *X. tropicalis* chromosomes were ~10% shorter in all cases (Fig. 3 B). Therefore, we conclude that although chromatin mass does influence spindle length, soluble cytoplasmic factors are the major determinant in *Xenopus* egg extracts.

To examine whether spindle size regulation is a static or dynamic process, we added fresh extract to preassembled spindles. To *X. tropicalis* extracts containing spindles that had incorporated X-rhodamine–labeled tubulin, we added three volumes of either *X. tropicalis* or *X. laevis* extract containing Alexa Fluor 488 tubulin, and examined spindle length at various time points after mixing. Whereas *X. tropicalis* extract did not affect spindle length over the course of the experiment, addition of *X. laevis* extract caused rapid growth of *X. tropicalis* spindles, by ~5 μm in length within 2 min, and to the size of premixed (75% *X. laevis*, 25% *X. tropicalis*) reactions within 5 min (Fig. 4 A). Reciprocally, the addition of *X. tropicalis* extract to preformed *X. laevis* spindles rapidly shrank them to the size of the premixed controls, whereas addition of *X. laevis* extract did not (Fig. 4 B). The added extract tubulin appeared to incorporate at the plus ends of growing microtubules in the central spindle (Fig. 4, A and B). These results demonstrate that soluble cytoplasmic factors dynamically govern spindle length in *Xenopus* extracts, in agreement with results obtained in *Drosophila melanogaster* cells (Goshima et al., 2005), and indicate that nonmicrotubule spindle matrix elements determining length, if they exist, cannot be purely static structures (Chang et al., 2004).

We found that chromatin bead spindles and asters induced by addition of DMSO or RanGTP were smaller in *X. tropicalis* extracts compared with *X. laevis* extracts (unpublished data). To determine if differences in global microtubule dynamics could account for the differences in spindle and aster size, we measured the parameters of microtubules nucleated from centrosomes in *X. laevis* and *X. tropicalis* extracts (Table I). Overall, although the microtubule growth rate was ~20% slower in the *X. tropicalis* extracts (14.7 μm/min vs. 18.5 μm/min in *X. laevis*; $P < 0.015$ in *t* test), catastrophe and rescue frequencies were similar, and the calculated steady-state microtubule lengths were not significantly different (Table I; Verde et al., 1992). Another mode of microtubule turnover in the spindle is poleward microtubule flux, which is when microtubules coordinately polymerize at their plus ends and depolymerize at their minus ends as antiparallel microtubules slide apart (Khodjakov and Kapoor, 2005). We measured these rates using speckle microscopy to mark the spindle microtubule lattice, and found values in both extracts similar to those previously described for *X. laevis*

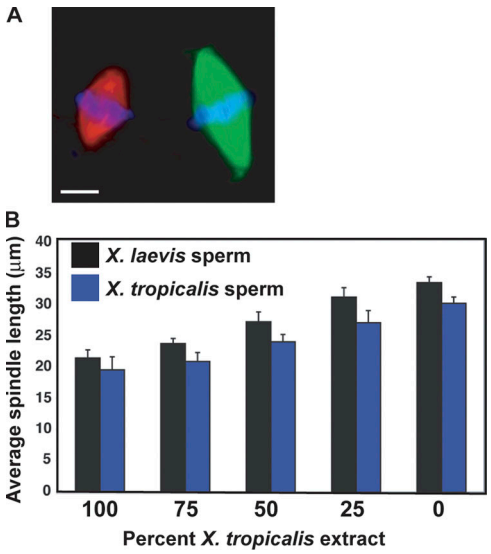

Figure 3. **Comparison of spindle length between *X. tropicalis* and *X. laevis*.** (A) Spindles assembled around *X. laevis* sperm nuclei in either *X. laevis* or *X. tropicalis* egg extracts were visualized using Hoechst dye (blue, DNA) and the incorporation of X-rhodamine tubulin (red microtubules, *X. tropicalis*), or Alexa Fluor 488 tubulin (green microtubules, *X. laevis*). Bar, 10 μm. (B) Mixed reactions with the indicated proportion of *X. tropicalis* extract were combined with *X. laevis* or *X. tropicalis* sperm nuclei. Spindle length was measured from pole to pole. A linear relationship was observed between the proportion of *X. laevis* extract present and spindle length. Error bars are the SD.

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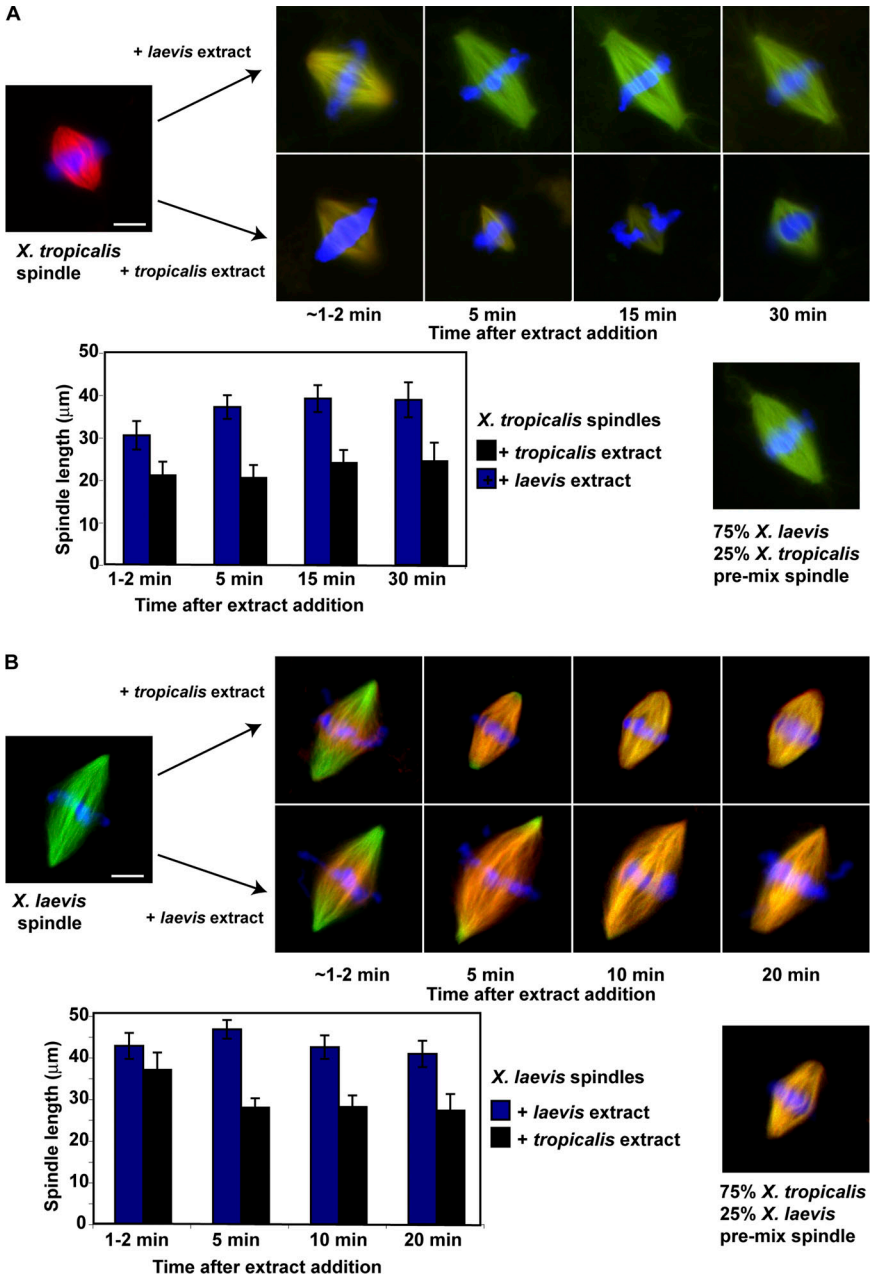

Figure 4. **Spindle length determination is highly dynamic in *Xenopus* extracts.** (A) Spindles assembled in *X. tropicalis* extracts containing X-rhodamine tubulin were diluted with three volumes of *X. laevis* or *X. tropicalis* extract supplemented with Alexa Fluor 488 tubulin, and fixed for examination at various time points. Quantification of spindle length over time after mixing revealed steady-state spindle lengths were reached within ~5 min, and they corresponded to those in the premixed control of 75% *X. laevis* 25% *X. tropicalis* extract. (B) The same experiment described in A, except that the spindles were assembled in *X. laevis* extract containing Alexa Fluor 488 tubulin, and diluted with *X. tropicalis* or *X. laevis* extract containing X-rhodamine tubulin. Quantification of spindle length over time after mixing is shown. Error bars are the SDs. Bars, 10 μm.

(Table I; 1.79 ± 0.33 μm/min for *X. laevis*; 2.25 ± 0. 25 μm/min for *X. tropicalis*; Desai et al., 1998). Thus, our results indicate that the coordinated microtubule sliding with balanced plus end polymerization and minus end depolymerization are not significantly different in the two extracts.

What is the underlying cause of spindle size difference in the two extracts? One possibility is that we are unable to make precise enough measurements to distinguish potentially causal differences in the dynamics of microtubules in the two extracts. Alternatively, other microtubule dynamic parameters, such as the frequency of nucleation, pausing, or severing, may generate differences in spindle size (Srayko et al., 2006). Although different in morphology, centrosomal microtubules in the two extracts grew to similar lengths, whereas microtubule structures induced by taxol, DMSO, and RanGTP were significantly smaller in *X. tropicalis* extracts (unpublished data). An intriguing possibility is that extract factors in the two systems respond differently to microtubule stabilizing/destabilizing agents, including mitotic chromatin. We think that this is possible because we have identified extract factors, such as the microtubule plus-end binding protein Xorbit, whose depletion does not obviously affect centrosomally nucleated microtubules, but strongly destabilizes spindle microtubules (Hannak and Heald, 2006). Extract-dependent changes in the activity of spindle factors or their regulation caused by a different kinase/phosphatase balance or RanGTP signal surrounding chromosomes could locally alter microtubule stability and overall spindle size. The challenge will now be to identify molecules that can account for the observed differences in spindle size in the egg extracts and compare the activities of the orthologous factors between the two *Xenopus* species.

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Table I. **Dynamic parameters of individual microtubules nucleated by centrosomes in *X. laevis* and *X. tropicalis* extracts and rates of poleward flux**

	X. laevis	*X. tropicalis*	*t* test
	n = 50	*n* = 79	
Growth rate (μm/min)	18.5; SD = 8.8	14.7; SD = 4.4	0.013
Shrinkage rate (μm/min)	21.8; SD = 9.3	17.6; SD = 5.0	0.19
Catastrophe frequency (events/min)	5.8; SD = 1.1	5.9; SD = 4.7	0.96
Rescue frequency (events/min)	3.0; SD = 2.6	4.3; SD = 4.9	0.44
Average length (μm)	5.7	6.3	0.42
Flux rates (μm/min)	1.79; SD = 0.33	2.25; SD = 0.25	0.31

Microtubule dynamics were measured from individual centrosomes in *X. laevis* and *X. tropicalis* CSF extracts. Dynamic parameters were computed using a custom algorithm. A *t* test was used to compute the significance of the differences. *n* denotes the number of individual microtubules measured for each extract system, taken from three different extracts. Average microtubule lengths were calculated according to Verde et al. (1992). Poleward flux rates were measured using time-lapse fluorescence speckle microscopy in 3 different extracts, with 5 spindles each (15 spindles total) for *X. laevis* and *X. tropicalis*.

In conclusion, *X. tropicalis* provides molecular advantages over *X. laevis* as a genetically and proteomically tractable system that can be applied to address cell biological questions using in vitro approaches. Although it could be expected that the smaller *X. tropicalis* eggs would have smaller spindles, our results show that this is because of a difference in cytoplasmic factors, rather than the size of the cell itself. By comparing cytoplasmic activities that are intrinsic to *X. laevis* and *X. tropicalis* extracts, new insights can be gained into mechanisms regulating cellular morphogenesis.

Materials and methods

Preparation of CSF extracts from *X. tropicalis*

CSF-arrested *X. tropicalis* extracts were prepared essentially as previously described for *X. laevis* (Murray, 1991; Desai et al., 1999), with the following exceptions. To induce egg laying, frogs were primed with 10 U of human chorionic gonadotropin (HCG) ~16 h before a hormone boost of 200 U HCG. Laying commenced ~4–5 h after the second HCG injection, and eggs were collected into water at 27–28°C (http://tropicalis.berkeley.edu/home/). Frogs were also squeezed ~6 h after the second HCG injection, and we found no substantial difference in the quality of the laid versus squeezed eggs. Pooled eggs were dejellied using 3% cysteine in water adjusted to pH 7.8 with NaOH. Incubation of frogs at temperatures below ~23°C inhibited egg laying, and resulted in poor extracts. Once the extract was prepared it was stored at 16°C, as extended storage at 4°C resulted in a loss of CSF arrest. Typical extract yield was 200–400 μl per frog.

Spindle size determination in mixed extract

X. laevis and *X. tropicalis* extracts were mixed in different proportions and supplemented with either *X. laevis* or *X. tropicalis* sperm nuclei prepared by standard procedures (Murray, 1991) at a concentration of 500 sperm/μl, and X-rhodamine tubulin at 0.125 mg/ml. Cycling reactions were performed, and reactions were diluted into spindle fix (30% glycerol, 1× BRB80, and 0.5% Triton X-100), spun onto coverslips, fixed in –20°C methanol, and mounted in Vectashield according to standard procedures (Desai et al., 1999). Images were collected with a fluorescence microscope (BX51; Olympus) and a cooled charge-coupled device camera (Orca II; Hamamatsu), and spindle lengths were measured using MetaMorph software (Molecular Devices). Spindle area measurements were made using thresholded images in MetaMorph. Mixing experiments were performed three independent times by three different investigators, and the results were averaged.

Immunoprecipitation and mass spectrometry of Xnf7

The Xnf7 polyclonal antibody was coupled to protein A dynabeads, as previously described (Maresca et al., 2005b). For immunoprecipitating Xnf7, 110 μl of either *X. tropicalis* or *X. laevis* CSF extract was subjected to three successive 45-min incubations on ice with anti-Xnf7–coated dynabeads. The beads from each round were pooled and washed extensively with XB before eluting for 5 min at room temperature into SDS sample buffer and retrieving the beads on a magnet. Half of the supernatant was subjected to SDS-PAGE, the gel was stained with Gradipure colloidal G-250 Coomassie blue stain (NuSep), and the indicated bands were excised and subjected to mass spectrometry at the University of California Berkeley Mass Spectrometry Facility.

Dynamics of spindle size determination

CSF reactions containing *X. laevis* sperm nuclei and 25 μl *X. tropicalis* extract (with X-rhodamine tubulin) or *X. laevis* extract (with Alexa Fluor 488 tubulin) were cycled through interphase and back into metaphase by the addition of 25 μl of the same type of extract (no sperm). Once metaphase spindles had assembled, the extract was split into two tubes, each containing 25 μl of the reaction mixture. As a control, 75 μl of the same type of extract supplemented with the other labeled tubulin was added to one of the tubes, while 75 μl of the opposing extract was added to the other 25-μl reaction. Each of the 100-μl reactions were quickly split into 4 separate tubes, and spindles from each condition were diluted and spun down onto coverslips as described in Spindle size determination in mixed extract for imaging and length measurements. All spindle reactions were incubated at ~23°C.

Microtubule dynamics and flux measurements

Centrosomes were prepared from KE37 cells as previously described (Chretien et al., 1997) and stored at −80°C. CSF extracts were prepared as described in the section Preparation of CSF extracts from *X. tropicalis*, and supplemented with either rhodamine tubulin (Cytoskeleton) or Alexa Fluor 488 tubulin (a gift from T. Whittman, University of California, San Francisco, San Francisco, CA) at 0.2 mg/ml. Centrosome reactions consisted of 8 μl of extract plus labeled tubulin, 1 μl of centrosomes, and 1 μl of Oxyrase. *X. laevis* extracts were incubated at ~20°C, and *X. tropicalis* extracts were incubated at ~23°C. To image centrosomes, 1 μl of extract was squashed under a 22-mm^2 coverslip and imaged using a 100×/1.3 NA objective. All glassware was base cleaned and stored in 95% ethanol until the time of use. Images were acquired every 3 s for 1–3 min. Microtubule lengths were measured if the microtubule could be followed for at least five successive frames. Microtubule lengths were measured as the distance from the center of the centrosome to the tip of the microtubule. Dynamics measurements were calculated using a custom-made spreadsheet (a gift from R. Tournebize, Institute Pasteur, Paris, France). Calculation of F_{cat} and F_{res} were made by manual inspection of raw growth and shrinkage measurements.

MT flux measurements were made using fluorescence speckle microscopy by incubation of cycled spindles with rhodamine-labeled tubulin at a concentration of 1 μg/ml (Kapoor and Mitchison, 2001). 2 μl of extract was gently squashed under a 22 × 22-mm coverslip previously outlined using a PAP pen. Images of speckles were collected every 5 s for 2 min using a 60×/1.4 NA objective. Speckle movements were tracked on kymographs to calculate the rate of flux. Measurements were made from at least five separate spindles from three different extracts for both *X. laevis* and *X. tropicalis*.

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