Chapter 14

Assays to Study Mitotic Centrosome and Spindle Pole Assembly and Regulation

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Abstract

Faithful chromosome segregation during cell division requires proper bipolar spindle assembly and critically depends on spindle pole integrity. In most animal cells, spindle poles form as the result of the concerted action of various factors operating in two independent pathways of microtubule assembly mediated by chromatin/RanGTP and by centrosomes. Mutation or deregulation of a number of spindle pole-organizing proteins has been linked to human diseases, including cancer and microcephaly. Our knowledge on how the spindle pole-organizing factors function at the molecular level and cooperate with one another is still quite limited. As the list of these factors expands, so does the need for the development of experimental approaches to study their function. Cell-free extracts from *Xenopus laevis* eggs have played an instrumental role in the dissection of the mechanisms of bipolar spindle assembly and have recently allowed the reconstitution of the key steps of the centrosome-driven microtubule nucleation pathway (Joukov et al., Mol Cell 55:578–591, 2014). Here we describe assays to study both centrosome-dependent and centrosome-independent spindle pole formation in *Xenopus* egg extracts. We also provide experimental procedures for the use of artificial centrosomes, such as microbeads coated with an anti-Aurora A antibody or a recombinant fragment of the Cep192 protein, to model and study centrosome maturation in egg extract. In addition, we detail the protocol for a microtubule regrowth assay that allows assessment of the centrosome-driven spindle microtubule assembly in mammalian cells.

Key words Mitosis, Spindle assembly, Spindle pole, Centrosome, Pericentriolar material, Microtubule-organizing center, Cep192, *Xenopus* egg extract, Cell-free system

1 Introduction

Spindle poles are inherent components of the mitotic/meiotic spindle and represent focal collections of microtubule (MT) minus ends towards which chromatids move during anaphase [1]. In addition to their role as centers of convergence of the chromosomes in nascent daughter cells, the spindle poles play two other important roles by maintaining spindle integrity in face of the forces of chromosome alignment and segregation [2, 3] and by anchoring spindle MTs, which is a prerequisite for chromosome segregation. Proper spindle pole assembly is, therefore, essential

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for accurate cell division and maintenance of genome stability. Indeed, supernumerary or malformed poles can lead to micronuclei formation and aneuploidy, which are the hallmarks of many cancers [2–4]. Not surprisingly, there is a growing list of spindle pole proteins implicated in cancer and other diseases [5].

In most animal cells, the position of each spindle pole is defined by the centrosome, a non-membrane-bound organelle that serves as the major MT-organizing center both in interphase and in mitosis. The centrosome consists of a pair of centrioles surrounded by pericentriolar material (PCM). Centrioles serve as templates for the assembly of new centrosomes and cilia and duplicate once per cell cycle (in S phase). The PCM nucleates and anchors MTs and participates in other centrosome functions. In addition to their role in spindle assembly, centrosomes are involved in a variety of other cellular processes, including motility, protein homeostasis, intracellular signaling, and immune response [6–8].

In the G2 phase of the cell cycle, the two centrosomes dramatically increase in size and MT-nucleating capacity owing to the recruitment of additional PCM components, such as the γ-tubulin ring complex (γ-TuRC), which serves as a MT template. This process, termed centrosome maturation, leads to the formation of two dense radial arrays of MTs (centrosomal MT asters) [6, 7]. Centrosome maturation and the consequent MT nucleation and anchoring are driven by a multistep Aurora A (AurA)-Plk1 kinase cascade locally organized by the key regulator of centrosome biogenesis, Cep192 [9, 10]. Notably, this cascade and the resulting focal MT nucleation can be recapitulated by artificial clustering of endogenous Cep192-Aur A-Plk1 complexes on the surface of protein-coated microbeads in metaphase (M-phase)-arrested *Xenopus* egg extract [10].

After the nuclear envelope breakdown, spindle MTs are also generated in the vicinity of chromatin by a completely different mechanism dependent on the small GTPase Ran. Specifically, Ran-GTP, which is produced by the chromatin-bound guanine nucleotide exchange factor for Ran, RCC1 [11] promotes bipolar spindle assembly by two cooperative mechanisms: (1) it releases multiple spindle assembly factors, such as TPX2, NuMA, HuRP, and NuSAP, from their inhibitory binding to importin α/β, thus promoting MT nucleation and growth, and (2) it enables the binding of certain other spindle assembly factors to exporting karyopherins (exportins), which is critical for the localization of these factors to specific spindle compartments [11]. The MTs generated by chromatin, centrosomes, and by two additional sources, kinetochores and MTs walls, are then organized by specialized proteins into a bipolar spindle [2, 3, 12].

Interestingly, although centrosomes are associated with and define the position of the spindle poles, they are not required for spindle assembly per se, since cells lacking these organelles (e.g., female meiotic cells) can form bipolar spindles and faithfully segregate chromosomes. Thus, the centrosome-independent pathways

of MT assembly suffice to assemble a functional bipolar spindle. Both the formation of acentrosomal spindle poles and the conversion of centrosomal MT asters into spindle poles were shown to require Ran-GTP and at least three groups of proteins: (1) MT minus end-associated spindle assembly factors (such as γ-TuRC and the MT crosslinking protein NuMA); (2) motor proteins (such as cytoplasmic dynein and kinesins); and (3) factors that affect MT growth and shrinkage (such as chTOG/XMAP215, katanin, and MT-destabilizing kinesins) [2, 3, 10, 13].

Our understanding of the mechanisms of spindle pole assembly has greatly benefited from studies in cell-free *Xenopus laevis* egg extracts. Such extracts are naturally arrested in M-phase of meiosis II and, when supplemented with demembranated sperm nuclei, form bipolar spindles [14, 15]. Moreover, since after the release of the arrest, the inherently oscillating activity of Cdk1/cyclin B drives cell cycle transitions in *Xenopus* egg extracts, this experimental system recapitulates (and allows the dissection of) both mitotic and interphase processes [15, 16]. The function of a protein of interest can be investigated in egg extracts by immunodepletion/reconstitution approaches or by the use of various inhibitors or dominant negative mutants. Studies in *Xenopus* egg extracts have allowed researchers to experimentally demonstrate the existence of and to recapitulate each of the four major independent pathways of spindle MT assembly, i.e., those mediated by the chromatin [17–21], kinetochores [22, 23], MT walls [24], and centrosomes [10].

This chapter primarily focuses on the application of *Xenopus* egg extracts to the analysis of mitotic centrosome and spindle pole assembly. Detailed protocols on the preparation of *Xenopus* egg extracts and demembranated sperm nuclei and on their use as tools to study spindle assembly can be found elsewhere [14–16, 25]. We first outline an assay for the analysis of acentrosomal spindle pole assembly. We then describe assays to model and study centrosome maturation using both natural (centrosomes) and artificial (beads coated with an anti-Aur A antibody or a recombinant Cep192 protein fragment) templates. In addition, we provide a protocol for the so-called MT regrowth assay that can be used to determine MT-nucleating activity of mitotic centrosomes in cultured mammalian cells.

2 Materials

2.1 Analysis of Acentrosomal Spindle Pole Assembly in Xenopus Egg Extracts

1. Recombinant human or *Xenopus* Ran(Q69L) protein. Protocols for preparation of recombinant Ran(Q69L) can be found elsewhere [26, 27] (*see* **Note 1**).
2. 1 M HEPES–KOH (pH 7.7). Dissolve HEPES in deionized water, adjust pH with KOH, filter-sterilize, and store at 4 °C (*see* **Note 2**).

3. 20× extract buffer (XB)-salts: 2 M KCl, 20 mM $MgCl_2$, 2 mM $CaCl_2$. Filter-sterilize and store at 4 °C.
4. 1.5 M sucrose. Dissolve in deionized water, filter-sterilize, and store at 4 °C.
5. 1 M dithiothreitol (DTT). Dissolve in deionized water, filter-sterilize, and store in 100 μL aliquots at −20 °C.
6. XB: 10 mM HEPES–KOH (pH 7.7), 100 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 50 mM sucrose, 1 mM DTT. Make fresh from the stock components without DTT (*see* **items 2–4**). Add DTT from a 1 M stock (*see* **item 5**) prior to use.
7. 0.5 M EDTA (pH 8.0). Add EDTA powder to deionized water and adjust pH with NaOH until the powder dissolves. Filter-sterilize and store at room temperature.
8. Guanosine 5′-triphosphate (GTP) sodium salt (Sigma-Aldrich).
9. 1 M $MgCl_2$. Dissolve in deionized water, filter-sterilize, and store at 4 °C.
10. 1.5 mL Eppendorf tubes.
11. 0.5 mL Eppendorf tubes.
12. Dialysis cassettes or dialysis tubing.
13. Protein concentrators, 0.5 mL, molecular-weight cutoff <10,000 kDa, low nonspecific binding (EMD Millipore Amicon or Thermo Scientific).
14. M-phase-arrested *Xenopus* egg extract. Detailed protocols for the preparation of the extract are described in refs. 14, 15 and 25. The extract should be quality tested before use (*see* **Notes 3** and **4**).
15. Water bath at 21 °C with a floating rack for microcentrifuge tubes (*see* **Note 5**).
16. Rhodamine-labeled tubulin (Cytoskeleton). Briefly spin the tubes and dissolve the content (20 μg of the lyophilized protein) in 2 μL of XB, to obtain a final concentration of 10 mg/mL. Snap-freeze the tubes in liquid nitrogen and store at −80 °C (*see* **Note 6**).
17. 10× MMR: 50 mM HEPES (pH 7.8), 1 M NaCl, 20 mM KCl, 10 mM $MgCl_2$, 20 mM $CaCl_2$, 1 mM EDTA (from a 0.5 M stock, *see* **item 7**). Dissolve the ingredients in deionized water, adjust pH with NaOH, filter-sterilize, and store at room temperature.
18. 16 % Formaldehyde in sealed ampoules (Ted Pella) (*see* **Note 7**).
19. 10 mg/mL Hoechst 33342/33258. Dissolve in deionized water and store at 4 °C.

20. Spindle fix solution: 50 % (v/v) glycerol, MMR (from a 10× stock, *see* **item 17**), 4 % formaldehyde (from a freshly opened 16 % stock, *see* **item 18**), 1 μg/mL Hoechst 33342/33258 (from a 10 mg/mL stock, *see* **item 19**). Make fresh prior to each experiment.
21. Microscope slides, 75 × 25 mm (Thermo Fisher Scientific) (*see* **Note 8**).
22. Coverslips, circular, 12 mm, # 1 (Thermo Fisher Scientific).
23. Straight fine-tip forceps (e.g., watchmaker forceps #5).
24. Nail polish.
25. Epifluorescent microscope.

2.2 Analysis of Centrosome Maturation in Xenopus Egg Extracts

1. 5 mg/mL Nocodazole (Sigma-Aldrich). Dissolve in DMSO and store in 50 μL aliquots at −20 °C.
2. Demembranated sperm nuclei (2×10^5/μL). Prepare as described in refs. 15 or 16.
3. 5× BRB-80: 400 mM PIPES (pH 6.8), 5 mM $MgCl_2$, 5 mM EGTA. Dissolve in deionized water and adjust pH with KOH. Filter-sterilize and store at 4 °C.
4. 20 % Triton X-100 (v/v). Dissolve in deionized water by mixing on a rotator or magnetic stirrer. Store at 4 °C.
5. Spindle dilution buffer: BRB-80 (from a 5× stock, *see* **item 3**), 30 % glycerol (v/v), 0.5 % Triton X-100 (from a 20 % stock, *see* **item 4**), 2 % formaldehyde (from a 16 % stock, *see* Subheading 2.1). Mix all ingredients except formaldehyde and store at room temperature for several weeks. Add formaldehyde just before use.
6. Cushion buffer: BRB-80 (from a 5× stock, *see* **item 3**), 40 % glycerol (v/v). Store at room temperature for several weeks.
7. BRB-80. Prepare from a 5× stock, *see* **item 3**.
8. 15 mL (18 × 102 mm) glass high strength centrifuge tubes (Kimble-Chase Kontes, Cat. #45500-15) equipped with custom-made adaptors and a metal hook or an equivalent tool for lifting the adaptors. These tools are described and schematically depicted in ref. 14.
9. Poly-d-lysine-coated coverslips, circular, 12 mm, #1 (Corning Biocoat, Cat. #354086).
10. Perforated holder with a lid for circular, 12 mm coverslips (made of an organic solvent-resistant plastic). An empty container from the Poly-d-lysine-coated circular coverslips (Corning Biocoat, Cat. #354086) ideally suits this purpose. Make a mark with a scratch on one side of the holder, to guide positioning of the inserted coverslips in the same direction (relative to the sample side).

11. Disposable plastic transfer pipets (1–2 mL).
12. High-speed refrigerated centrifuge with an HB-6 (Sorvall) or equivalent rotor.
13. Glass jar with a lid for microscope slides filled with methanol at −20 °C. Store closed in a freezer for up to 2 weeks. Replace methanol after each experiment.
14. Cell culture plates, 24 wells.
15. Parafilm.
16. 1 M Tris–HCl (pH 7.5). Dissolve Tris base in deionized water, adjust pH to 7.5 with concentrated HCl, and store at room temperature.
17. TBS-T: 25 mM Tris–HCl (pH 7.5) (from a 1 M stock, *see* **item 16**), 150 mM NaCl, 0.05 % Tween 20.
18. TBS-T/5 % goat serum: Add goat serum to TBS-T (*see* **item 17**), mix, and filter through a 2-μM filter. Prepare fresh before each experiment.
19. Pasteur pipettes.
20. Vacuum aspirator.
21. Aluminum foil.
22. Specific antibodies for proteins of interest.
23. Fluorochrome-labeled secondary antibody(ies).
24. Vectashield Mounting Medium with DAPI (Vector Laboratories).
25. Cycling egg extract. Prepare as described in detail in ref. 15 (*see* **Note 9**).

2.3 Using Anti-Aur A Antibody-Coated Beads to Model and Study Mitotic Centrosome Formation

1. Dynabeads Protein A (Life Technologies).
2. 0.5 mL siliconized Eppendorf tubes.
3. Variable speed vortex mixer.
4. Magnetic particle concentrator for microtubes referred to in the text as "magnet" (Dynal/Life Technologies).
5. Phosphate-buffered saline (PBS): 140 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4 (pH 7.4). Prepare by dissolving a commercially available stock solution or powder in deionized water and store at 4 °C.
6. 50 mg/mL bovine serum albumin (BSA), molecular biology grade. Dissolve in PBS (*see* **item 5**) and store in 1 mL aliquots at −20 °C. The aliquots can be repeatedly frozen/thawed.
7. PBS/2 mg/mL BSA/0.05 % Tween 20. Prepare before the experiment by mixing PBS (*see* **item 5**), BSA (from a 50 mg/mL stock, *see* **item 6**), and Tween 20.
8. Microcentrifuge.

9. Affinity purified rabbit polyclonal anti-AurA antibody (αAurA) [9]. Dilute in PBS to obtain a concentration of 1 mg/mL (*see* **Note 10**).
10. Variable speed rotator (such as Cole-Parmer Roto-Torque Rotator).
11. 0.2 M sodium borate buffer (pH 8.5)/0.02 % Tween 20. Prepare by dissolving/diluting a borate buffer stock (Thermo Scientific) and adding Tween 20.
12. Dimethyl pimelimidate (DMP), 50 mg vials individually sealed in moisture-proof packages (Thermo Scientific).
13. Crosslinking stop buffer: 100 mM Tris–HCl (pH 7.5) (from a 1 M stock, *see* Subheading 2.2), 150 mM NaCl, 0.05 % Tween 20.
14. PBS/0.1 % Tween 20. Add Tween 20 to PBS (*see* **item 5**).
15. PBS-500/0.1 % Tween 20. Add 210 mg of NaCl to 10 mL of PBS/0.1 % Tween 20 (*see* **item 14**) and mix until NaCl dissolves.
16. 20 % sodium azide. Dissolve in water and store at room temperature (highly toxic when ingested or inhaled).
17. XB/1 mg/mL BSA. Make fresh for each experiment by adding BSA (from a 50 mg/mL stock, *see* **item 6**) to XB (*see* Subheading 2.1). Keep on ice.
18. XB/1 mg/mL BSA/0.05 % Tween 20. Add Tween 20 to the XB/1 mg/mL BSA (*see* **item 17**), mix, and keep on ice.
19. XB/0.1 % Tween 20. Add Tween 20 to XB (*see* Subheading 2.1), mix, and keep on ice.

2.4 Using Cep192 Beads to Model and Study Mitotic Centrosome Formation

1. LB medium: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl. Dissolve in deionized water and sterilize by autoclaving.
2. *E. coli* cells expressing $Cep192^{1–1000}$-GST fusion proteins. Transform *E. coli* cells optimized for protein expression (e.g., BL21) with a cDNA encoding $Cep192^{1–1000}$ wild type (wt) ($Cep192^{1–1000}$-wt) or its mutants of interest in a pGEX vector (GE Healthcare) [9, 10].
3. Temperature-controlled shaker incubator for flasks.
4. Laboratory flasks (100 mL–2 L).
5. UV–visible-wavelength spectrophotometer.
6. 1 M Isopropyl-β-d-thiogalactopyranoside (IPTG). Dissolve in deionized water and store at −20 °C.
7. 1 M Tris–HCl (pH 8.0). Dissolve Tris base in deionized water, adjust pH with concentrated HCl, and store at room temperature.
8. Protease inhibitor cocktail (e.g., cOmplete protease inhibitor cocktail tablets from Roche Life Science).

9. NETS buffer: 20 mM Tris–HCl (pH 8.0) (from a 1 M stock, *see* **item 7**), 100 mM NaCl, 1 mM EDTA (from a 0.5 M stock, *see* Subheading 2.1), 10 % glycerol (v/v), 1.5 % Sarkosyl (*N*-Lauroylsarcosine), 5 mM β-mercaptoethanol. Store at 4 °C. Prior to use, add protease inhibitors [e.g., 1 tablet of cOmplete (Roche Life Science) per 25 mL].
10. 15 mL Falcon tubes.
11. Lysozyme powder (Sigma-Aldrich). Store at −20 °C.
12. Sonicator (with a probe).
13. Refrigerated microcentrifuge.
14. NETN buffer: 20 mM Tris–HCl (pH 8.0) (from a 1 M stock, *see* **item 7**), 100 mM NaCl, 1 mM EDTA (from a 0.5 M stock, *see* Subheading 2.1), 10 % glycerol, 0.5 % (v/v) Nonidet P-40 (NP-40), 5 mM β-mercaptoethanol. Store at 4 °C.
15. NETN buffer/500 mM NaCl. Dissolve 232 mg of NaCl in 10 mL of NETN buffer (*see* **item 14**).

2.5 Analysis of Centrosome-Driven Spindle MT Assembly in Mammalian Cells

1. Coverslips, 22 mm × 22 mm, #1.
2. Cell culture dishes, 35-mm or 60-mm.
3. Dulbecco's Modified Eagle Medium (DMEM) containing 2 mM l-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Store sterile at 4 °C.
4. DMEM/10 % fetal bovine serum (FBS). Add FBS to DMEM (*see* **item 3**). Store sterile at 4 °C.
5. Humidified incubator at 37 °C, 5 % CO_2.
6. 10 mM RO-3306 (EMD Millipore). Dissolve in DMSO and store in aliquots at −20 °C.
7. 10 mM MG-132 (Boston Biochem). Dissolve in DMSO and store in aliquots at −20 °C.
8. Ice water bath. Fill an ice bucket with ice and add water until the top layer of the ice is covered.
9. Water bath at 37 °C.
10. Methanol. Chill to −20 °C before the experiment.

3 Methods

3.1 Analysis of Acentrosomal Spindle Pole Assembly in Xenopus Egg Extracts

Accumulated evidence has revealed a key role of Ran-GTP in bipolar spindle assembly [11]. Indeed, the formation of bipolar spindles can be recapitulated in M-phase-arrested *Xenopus* egg extract independently of chromatin and centrosomes by either adding DNA-coated beads (which mimic chromatin and recruit RCC1) [11, 18, 21], or simply increasing the concentration of RanGTP [20, 21].

The addition of a constitutively active Ran mutant defective in GTP hydrolysis [Ran(Q69L)-GTP] to the extract promotes the formation of MT asters and spindles with focused poles akin to those in acentrosomal meiotic spindles [21]. Moreover, in this setting, spindle pole focusing depends on the function of proteins known to be implicated in the formation of acentrosomal spindle poles [27, 28] (Fig. 1). Thus, the RanGTP-induced MT assembly assay can be used as a simplified tool for the dissection of the

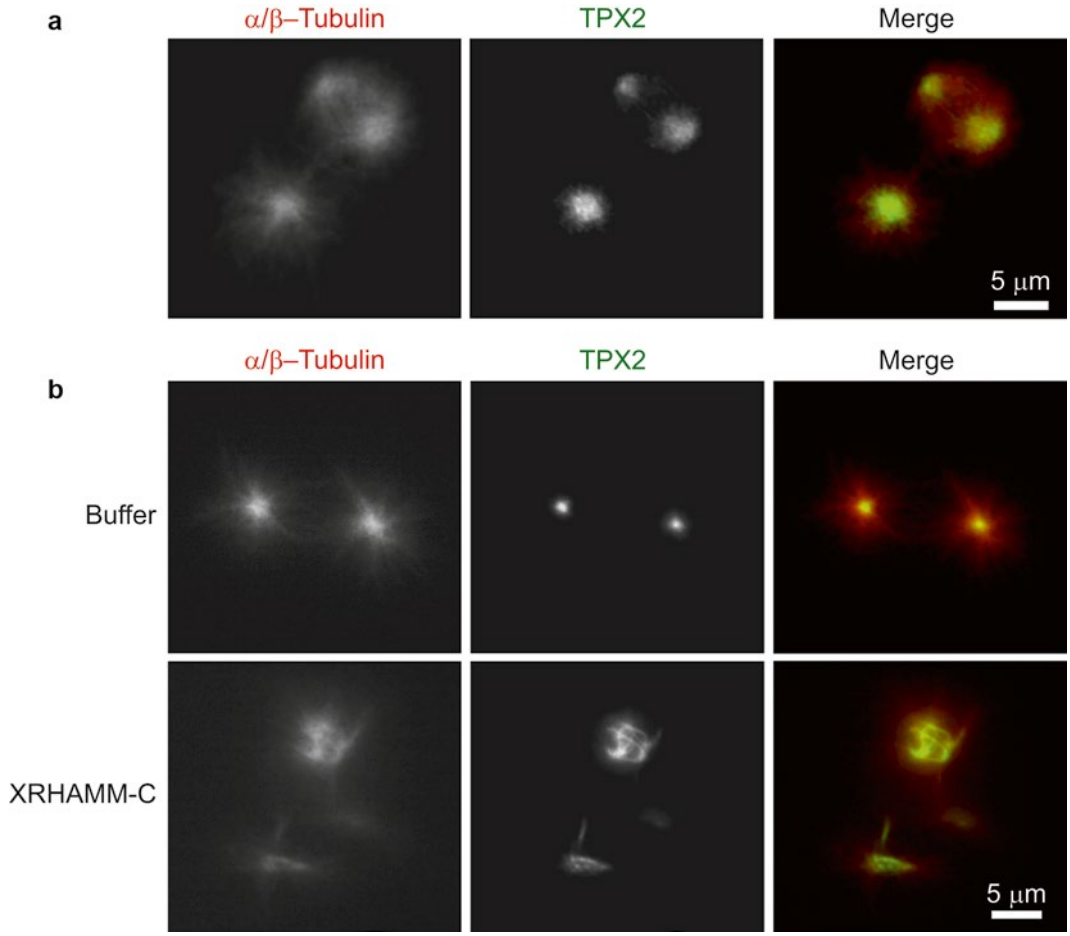

Fig. 1 RanGTP-induced MT asters and spindles formed in M-phase-arrested *Xenopus* egg extract. (**a**) The extract was supplemented with rhodamine-labeled tubulin and 15 μM of Ran(Q69L)-GTP and incubated at 21 °C for 20 min. MT structures were spun down onto a coverslip, stained with an anti-TPX2 antibody, and analyzed by immunofluorescence. A representative aster and a spindle are shown. (**b**) The extract was supplemented with rhodamine-labeled tubulin, a trace amount (5 μg/mL) of an Alexa Fluor 488-labeled anti-TPX2 antibody, and with either XB (*upper panels*) or a recombinant C-terminal fragment of XRHAMM (XRHAMM-C) [27], which is a protein partner of TPX2 in spindle assembly [28]. After the addition of 15 μM of Ran(Q69L)-GTP followed by the incubation at 21 °C for 20 min, the extracts were analyzed by fluorescence microscopy. Note the disruption of the poles of MT asters in the XRHAMM-C-supplemented extract, owing to the interference of XRHAMM-C with the function of endogenous XRHAMM [27]

mechanisms and factors involved in acentrosomal spindle pole assembly. Below, we describe protocols for the preparation of Ran(Q69L)-GTP (Subheading 3.1.1) and for the analysis of RanGTP-promoted spindle MT assembly (Subheading 3.1.2). The RanGTP-induced MT structures can be visualized by fluorescence microscopy of the extracts supplemented with rhodamine-labeled tubulin (Subheading 3.1.2) or by immunofluorescence as described below for the centrosomal MT asters (Subheading 3.2.2). A detailed protocol for the use of DNA-coated beads to study acentrosomal spindle assembly can be found in ref. 14.

3.1.1 Preparation of Ran(Q69L)GTP

1. Dialyze recombinant Ran(Q69L) against two changes of XB and concentrate to ~5–20 mg/mL (*see* **Note 1**).
2. Add 10 μL of 0.5 M EDTA to 1 mL of the Ran(Q69L) solution (yielding a final EDTA concentration of 5 mM) and mix by pipetting up and down several times.
3. Weight out 10–40 mg of GTP in a new 1.5 mL Eppendorf tube and add the Ran(Q69L) solution from **step 2** to it (*see* **Note 11**). Mix gently by inverting the tube or by pipetting up and down until the powder dissolves. Spin briefly and place the tube on ice for 1 h.
4. Add 50 μL of 1 M $MgCl_2$ to the tube, mix, and dialyze the mixture against two changes of XB at 4 °C for 2 h each.
5. Concentrate Ran(Q69L)-GTP to ~10–20 mg/mL (~0.4–0.8 mM) and snap-freeze in 3–5 μL aliquots in liquid nitrogen. Store at −80 °C.

3.1.2 RanGTP-Induced Spindle MT Assembly in Xenopus Egg Extracts

1. Thaw an aliquot of the M-phase-arrested *Xenopus* egg extract in a water bath at 21 °C and immediately place it on ice.
2. Add rhodamine-labeled tubulin to the extract to a final concentration of ~50–75 ng/μL, mix gently by pipetting up and down (*see* **Note 12**), and place the extract on ice until use (*see* **Note 13**).
3. Pipet 10 μL of each extract sample to a new 0.5 mL Eppendorf tube and add Ran(Q69L)-GTP to a final concentration of 15 μM (*see* **Note 14**). Mix by pipetting up and down and place the tube(s) in a water bath at 21 °C.
4. At the desired time intervals (~5–40 min) (*see* **Note 15**), pipet onto a microscope slide(s) 1.2 μL-drops (up to 6 drops per slide) of the spindle fix solution in a number corresponding to the number of extract samples. To each drop, add 0.8 μL of egg extract from **step 3**.
5. Using straight fine-tip forceps, gently lower a circular 12 mm coverslip on each drop of the fixed extract sample. After the fluid has evenly distributed under the coverslips, seal the edges

of each coverslip with nail polish. Avoid moving the coverslips. Air-dry and store the slides with fixed extract samples in the dark at room temperature (up to 1 month).

6. Examine the samples by fluorescence microscopy (*see* **Note 16**).

3.2 Analysis of Centrosome Maturation in Xenopus Egg Extracts

Analysis of centrosome assembly and function can be carried out in *Xenopus* egg extracts supplemented with demembranated sperm nuclei. Each sperm nucleus contains a pair of "naked" (i.e., almost devoid of PCM) centrioles attached to it. When sperm nuclei are added to the extract, the centrioles recruit PCM components and, depending on the cell cycle phase of the extract, form either a mitotic or an interphase centrosome. Centrosome maturation (which represents a transition of the centrosome from an interphase to a mitotic state) can be recapitulated and studied in the so-called cycling egg extracts, which oscillate between S and M phases due to the inherent periodic changes in the activity of the Cdk1/cyclin B complex in the egg cytoplasm [15]. The preparation and handling of cycling extracts is, however, a daunting task. Thus, it is more practical to model and study centrosome maturation in M-phase-arrested egg extracts. The experimental conclusions can then be verified in cycling extracts or cultured cells.

When a naïve M-phase-arrested egg extract is supplemented with sperm nuclei, the centrioles rapidly recruit PCM components and within minutes form a mitotic centrosome that nucleates a MT aster (Fig. 2, upper panels). Importantly, all these processes initially occur independently of the chromatin/Ran-GTP-dependent pathway of MT assembly, which becomes active only at later time points.

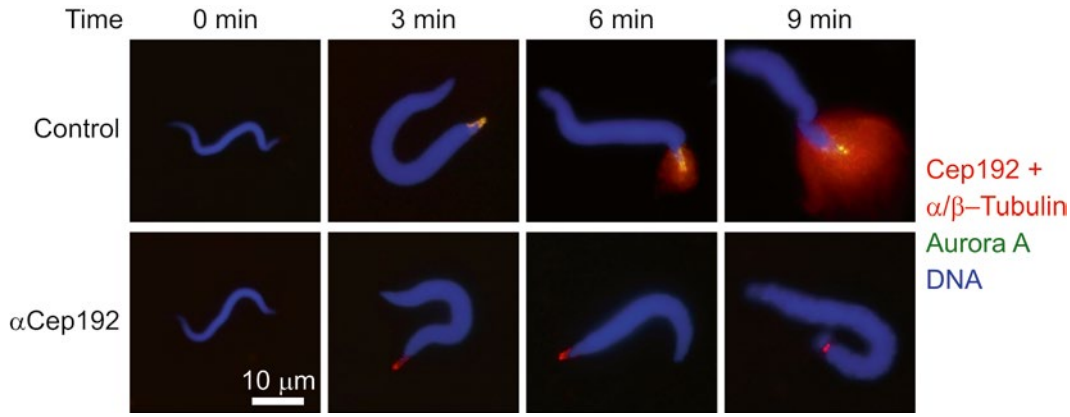

Fig. 2 Time course of the recruitment to the sperm nuclei-associated centrioles of Cep192 and AurA and of centrosome-driven MT assembly in M-phase-arrested *Xenopus* egg extract. The extract was supplemented with 100 μg/mL of non-immune IgG (*upper panels*) or αCep192 (*lower panels*), an N-terminally directed anti-Cep192 antibody that inhibits Cep192 function, in part by interfering with the binding of AurA [9]. Note that the targeting of AurA to centrosomes and the centrosome-driven MT assembly are compromised in the presence αCep192. MTs and Cep192 are in *red*, AurA is in *green*, and DNA is in *blue*. Colocalization of AurA and Cep192 produced a *yellow* signal (Reprinted with permission from Ref. 33, copyright 2011)

Below, we describe a protocol for the analysis of centrosome maturation in M-phase-arrested extract in two steps: setting up and monitoring the mitotic centrosome assembly reactions (Subheading 3.2.1) and preparing the samples for immunofluorescence (Subheading 3.2.2). We then outline the procedure for the analysis of centrosome maturation in cycling egg extracts (Subheading 3.2.3). A protocol for the preparation of such extracts is described in ref. 15 (*see* **Note 9**).

3.2.1 Setting Up and Monitoring the Mitotic Centrosome Assembly Reactions in M-Phase-Arrested Xenopus Egg Extracts

1. Thaw an aliquot of the M-phase-arrested extract (Subheading 2.1) and place it on ice.
2. Add to the extract either nocodazole to a final concentration of 15 μg/mL (for the analysis of PCM protein recruitment) (*see* **Note 17**) or rhodamine-labeled tubulin (Subheading 2.1) to a final concentration of ~50–75 ng/μL (for the analysis of centrosome-driven MT assembly) and mix by pipetting up and down.
3. Thaw an aliquot of demembranated sperm nuclei and immediately place it on ice.
4. Pipet 10–30 μL of each egg extract sample (*see* **Note 18**) to a 0.5 mL Eppendorf tube and place the tubes on ice.
5. To initiate the centrosome assembly reactions, add sperm nuclei to a final concentration of 1.5×10^3/μL to the first extract sample, mix by pipetting up and down, and place the tube in a 21 °C water bath (Subheading 2.1). Repeat this procedure for the other extract samples with the same time interval.
6. To monitor the formation of centrosomal MT asters, at the desired time points (2–12 min, *see* **Note 19**) and with the same time interval between the samples as that in **step 5**, withdraw 0.8-μL aliquots of the rhodamine-tubulin-supplemented extracts and analyze by fluorescence microscopy as described in Subheading 3.1.2, **steps 4–6**.

3.2.2 Preparing Samples for Immunofluorescence

1. Place 1.5 mL Eppendorf tubes (in a number corresponding to the number of extract samples multiplied by the number of time points to be analyzed) on a rack at room temperature. Add 0.5 μL of spindle dilution buffer to each tube.
2. At a selected time interval(s) (*see* **Notes 18** and **19**), withdraw 5–10 μL of extract from each sample in Subheading 3.2.1, **step 5**, and add to the corresponding 1.5 mL tube with spindle dilution buffer. Mix the tube immediately by inverting and incubate at room temperature for 10 min.
3. During the incubation, prepare custom-modified spin-down glass centrifuge tubes with adaptors [14] in the number corresponding to the number of the diluted fixed extract samples in

step 2. To each tube, add 4 mL of cushion buffer and a circular, 12 mm, poly-d-lysine-coated coverslip. Using a metal hook, submerge the coverslip, placing it horizontally on top of the adaptor.

4. Using a disposable plastic transfer pipet, layer each diluted fixed extract sample onto the cushion buffer.
5. Spin down the tubes at 10,000 rpm (~16,000 × *g*) in an HB-6 rotor for 15 min at 16 °C (*see* **Note 20**).
6. Place the tubes on a rack and aspirate the fluid leaving a ~1 cm layer. Using a disposable plastic transfer pipet, slowly add ~4 mL of BRB-80 to each tube along the tube's wall. Aspirate BRB-80, leaving a ~0.5 cm layer of the cushion buffer above the coverslip.
7. While wearing gloves, use a metal hook to carefully lift the adaptor towards the top of the tube. When the adaptor has emerged approximately half-length out of the tube, press it with a finger against the wall of the tube and hold. Using fine-tip forceps, lift the coverslip and insert it into a slot of the perforated holder for circular, 12 mm coverslips. Make sure that the sample side of the coverslip is directed towards the mark on the holder. Repeat this step for the other spun-down samples.
8. Close the coverslip holder with a lid and place it into a glass jar for microscope slides filled with methanol at –20 °C. Incubate for 5 min.
9. Remove the coverslip holder from methanol and place it on a paper towel. Open the holder and, using fine-tip forceps, transfer each coverslip, sample side up, to a well of a 24 well cell culture plate filled with ~1.5 mL of TBS-T. Aspirate TBS-T and refill the wells with TBS-T. Cover the 24 well cell culture plate with a lid, seal with Parafilm, wrap in aluminum foil, and store at 4 °C until immunofluorescence analysis (up to several weeks).
10. Aspirate TBS-T from the wells with coverslips and wash the coverslip twice with ~1.5 mL of TBS-T. For each wash, fill the wells with TBS-T directing the fluid stream at the wall of the well. Gently swirl the plate and leave it on the bench for 2 min. Aspirate TBS-T.
11. Add to each well 0.5 mL of TBS-T/5% goat serum and incubate at room temperature for 10 min.
12. Dilute the primary antibody to the recommended concentration in TBS-T/5 % goat serum (~50–75 µL per coverslip).
13. Lay out a sheet of Parafilm that is slightly larger in size than the 24 well plate on a clean flat surface (*see* **Note 21**).
14. For each coverslip, pipet ~50–75 µL of the diluted primary antibody on Parafilm. Make sure that the distance between the drops of antibody solution is at least 3–4 cm.

15. Using fine-tip forceps, take the first coverslip from **step 11** and remove excess fluid by gently blotting the reverse side and edges of the coverslip with a Kimwipes. Place the coverslip, sample side down, onto the antibody drop. Repeat this step for the remaining coverslips.
16. Cover the bottom of the lid of the cell culture dish with a stack of ~3–5 layers of Kimwipes and spray with distilled water from a wash bottle. Gently tap down to ensure that the Kimwipes are attached to the lid and remove the excess of water with dry Kimwipes. Tightly cover the coverslips on the Parafilm with the lid. If a fluorochrome-labeled antibody is used, cover the lid with aluminum foil. Incubate at room temperature for 1 h.
17. Squirt ~0.5 mL of TBS-T under each coverslip to lift it up. Remove the coverslips from the Parafilm and transfer them to the wells of a 24 well cell culture plate filled with TBS-T.
18. Wash the coverslips in TBS-T three times, 3 min each, at room temperature.
19. For indirect immunofluorescence, repeat **steps 13–18** using, instead of the primary antibody, a fluorochrome-labeled secondary antibody diluted to an appropriate concentration in TBS-T.
20. Apply ~20 μL drops of Vectashield Mounting Medium with DAPI onto a microscope slide (up to 6 drops per slide). Using fine-tip forceps, take each coverslip from the 24 well plate and remove excess fluid by gently blotting the reverse side and edges of the coverslip with a Kimwipes. Place the coverslip, sample side down, onto the drop of Vectashield.
21. To remove the excess of Vectashield, place a stack of 3–5 layers of Kimwipes on top of the microscope slide and cover with 3–5 paper towels. Apply gentle pressure on the paper towels avoiding sideways movements.
22. Seal the margins of the coverslips with nail polish and allow to air-dry. Gently remove residual salts from the coverslip with a wet Kimwipes and air-dry.
23. Store slides in the dark at 4 °C until imaging.

3.2.3 Analysis of Centrosome Maturation in Cycling Egg Extracts

1. Prepare cycling egg extract as described in ref. 15 and supplement it with rhodamine-labeled tubulin (Subheading 2.1) (*see* **Note 9**).
2. Pipet 50 μL of each extract sample into a 0.5 mL Eppendorf tube and add sperm nuclei (Subheading 2.2) to a final concentration of $1 \times 10^3/\mu L$. Mix gently by pipetting up and down and place the tube in a water bath at 21 °C (Subheading 2.1).
3. Every 10–15 min (*see* **Note 22**), withdraw two aliquots from each extract sample: a 0.5–1 μL aliquot for the analysis by SDS-PAGE/Western blotting and a 0.8 μL aliquot for moni-

toring chromatin and MT dynamics by fluorescence microscopy, as described in Subheading 3.1.2, **steps 4–6**.

4. After large nuclei have formed in the extract, watch for the signs of centrosome maturation, as evident by the appearance of MT asters, which often (but not always) neighbor the nuclei.
5. After detecting centrosomal MT asters in the control (mock) extract sample, withdraw a 10 μL aliquot from each extract sample and analyze by immunofluorescence as described in Subheading 3.2.2. If necessary, withdraw an additional aliquot(s) of each extract with a ~15–20 min interval for immunofluorescence analysis.
6. Analyze aliquots of the extracts from **step 3** by Western blotting of the PCM proteins of interest and of the markers of cell cycle progression (such as cyclin B and phosphotyrosine 15 of Cdk1/Cdc2).

3.3 Using Anti-Aur A Antibody-Coated Beads to Model and Study Mitotic Centrosome Formation

Centrosome maturation is driven by a multistep Cep192-organized signaling cascade, in which Cep192 serves both as an activating scaffold for AurA and Plk1 and as an anchoring platform for Plk1 phosphorylation-mediated γ-TuRC recruitment [10]. The cascade is initiated by AurA autophosphorylation at T288/T295 (in human/*Xenopus* AurA, respectively), which results from the recruitment and consequent accumulation and clustering of the Cep192/AurA/Plk1 complexes at centrosomes. AurA then activates Plk1 by phosphorylating it at T210/T201 (in human/*Xenopus* Plk1, respectively) in the T-loop. Plk1, in turn, phosphorylates Cep192 to generate the binding sites for γ-TuRC and, possibly, other PCM components, thus promoting MT nucleation [10]. Notably, γ-TuRC recruitment and MT nucleation can be mimicked in egg extract independently of the centrosomal milieu, by local oligomerization/clustering of endogenous Cep192 complexes on the surface of beads coated with a bivalent αAurA. In this case, Cep192 (all of which is bound to AurA in egg cytoplasm) is co-recruited with AurA to the surface of αAurA beads. The αAurA-mediated dimerization of Cep192-AurA complexes then promotes AurA autophosphorylation at T295, thus triggering the Cep192-organized AurA-Plk1 cascade [9, 10]. Hence, αAurA beads act as artificial centrosomes and can be used to model and study centrosome maturation at steps downstream of the Cep192-dependent AurA activation. The centrosome-like behavior of αAurA beads in egg extract was first reported by Tsai and Zheng [13] and the mechanism of this phenomenon was subsequently elucidated by Joukov and colleagues [9, 10]. Below, we describe the procedure for the preparation of αAurA beads (Subheading 3.3.1) followed by the protocols for the application of αAurA beads to the modeling of centrosome-driven MT assembly (Subheading 3.3.2) and PCM protein recruitment (Subheading 3.3.3).

3.3.1 Preparing αAurA Beads

1. Resuspend Dynabeads Protein A thoroughly by vortexing the stock vial and transfer 0.2 mL of the beads to a new 0.5 mL siliconized Eppendorf tube (*see* **Note 23**). Place the tube on the magnet for 1 min and pipet off the supernatant.
2. Remove the tube from the magnet, add 0.4 mL of PBS/2 mg/mL BSA/0.05 % Tween 20, and resuspend the beads by vortexing. Briefly spin the tube, place it on the magnet for 1 min, and pipet off the supernatant.
3. Wash the beads two more times, with 0.4 mL and 0.2 mL of PBS/2 mg/mL BSA/0.05 % Tween 20 (*see* **Note 24**).
4. Remove the tube from the magnet and add 0.2 mL of αAurA (1 mg/mL). Mix the beads by pipetting up and down and place the tube on a rotator at 4 °C for 2 h.
5. Remove the tube from the rotator, spin it briefly, and place on the magnet for 1 min. Transfer the supernatant to a clean 0.5 mL tube and measure the concentration of IgG in the supernatant. Add 0.5 μL of sodium azide to the αAurA supernatant and store at 4 °C (*see* **Note 25**).
6. Resuspend the beads in 0.2 mL of 0.2 M sodium borate buffer (pH 8.5)/0.02 % Tween 20 at room temperature and transfer to a clean 1.5 mL Eppendorf tube. Bring the volume to 1 mL with the same buffer. Vortex the tube, spin it briefly, and place on the magnet for 1 min. Pipet off the supernatant.
7. Add 1 mL of 0.2 M sodium borate buffer (pH 8.5)/0.02 % Tween 20 to the tube with the beads, vortex, and leave at room temperature.
8. Transfer a 20 μL aliquot of the beads to a new 0.5 mL siliconized Eppendorf tube. Place the 0.5 mL Eppendorf tube on a magnet for 1 min, remove the supernatant, and add 20 μL of SDS-PAGE sample buffer to the tube. Analyze this (pre-crosslinked) αAurA bead sample in **step 13**.
9. Bring a sealed package with a vial of DMP to room temperature. Open the package and the vial and weight out 6.5 mg of DMP on a weigh paper. Immediately add the DMP powder to the 1.5 mL Eppendorf tube with αAurA beads in **step 7**, close the tube, and invert it several times to dissolve DMP. Place the tube on a rotator and incubate at room temperature for 45 min (*see* **Note 26**).
10. Vortex the 1.5 mL tube with αAurA beads and transfer a 20 μL aliquot to a clean 0.5 mL siliconized Eppendorf tube. Place the 0.5 mL Eppendorf tube on the magnet for 1 min, remove the supernatant, and add 20 μL of SDS-PAGE sample buffer to the tube. Analyze this (post-crosslinked) αAurA bead sample in **step 13**.

11. Briefly spin the 1.5 mL tube with αAurA beads and place it on the magnet for 1 min. Remove the supernatant and wash the beads twice in 1 mL of the crosslinking stop buffer (*see* Subheading 2.3). Add 1 mL of the crosslinking stop buffer to the beads, vortex, and leave the tube at 4 °C overnight.
12. Briefly spin the 1.5 mL tube with αAurA beads and place it on the magnet for 1 min. Pipet off the supernatant and wash the beads twice with 1 mL of PBS-500/0.1 % Tween 20 and three times with 1 mL of PBS/0.1 % Tween 20. Resuspend the beads in 200 μL of PBS/0.1 % Tween 20 and transfer to a new 0.5 mL siliconized Eppendorf tube. Add 0.5 μL of 20 % sodium azide to the tube and vortex. Spin the tube briefly, seal it with Parafilm, and store at 4 °C.
13. To assess the efficiency of the crosslinking of αAurA to the Dynabeads Protein A, run the pre-crosslinked and post-crosslinked bead samples from **steps 8** and **10**, together with a protein molecular weight marker, on a SDS-PAGE gel followed by staining the gel with a protein-binding dye (e.g., Coomassie Blue).

3.3.2 Analysis of αAurA Bead-Induced MT Assembly

1. Thaw an aliquot of the M-phase-arrested extract and add to it rhodamine-labeled tubulin (Subheading 3.1.2, **steps 1** and **2**).
2. Vortex the stock tube of αAurA beads (Subheading 3.3.1) and pipet 2 μL to a 0.5 mL siliconized tube containing 200 μL of XB/1 mg/mL BSA/0.05 % Tween 20. Wash the beads twice in 200 μL of XB/1 mg/mL BSA/0.05 % Tween 20 and once in 50 μL of XB/1 mg/mL BSA. Place the tube on the magnet for 1 min, pipet off the supernatant, and resuspend the beads in 10 μL of XB/1 mg/mL BSA.
3. Pipet 15 μL of the rhodamine-tubulin-supplemented extract from **step 1** into a clean 0.5 mL siliconized Eppendorf tube and add 0.5 μL of αAurA beads from **step 2**. Mix by pipetting up and down and place the tube on ice for 30 min. Mix the extract gently every 5–10 min by flicking the tube or by swirling with a pipette tip.
4. Briefly spin the tube and add to the extract Ran(Q69L)-GTP (Subheading 3.1.1) to a final concentration of 8 μM. Mix by pipetting up and down and place the tube in a 21 °C bath (*see* **Note 27**).
5. Every 5 min of the incubation, gently flick the tube to mix the beads and take a 0.8 μL aliquot for the analysis by fluorescence microscopy as described in Subheading 3.1.2, **steps 4–6**. The αAurA bead-induced MT asters and spindles form after ~5–30 min (depending on the quality of the extract) incubation of the extract at 21 °C (Fig. 3a, b).

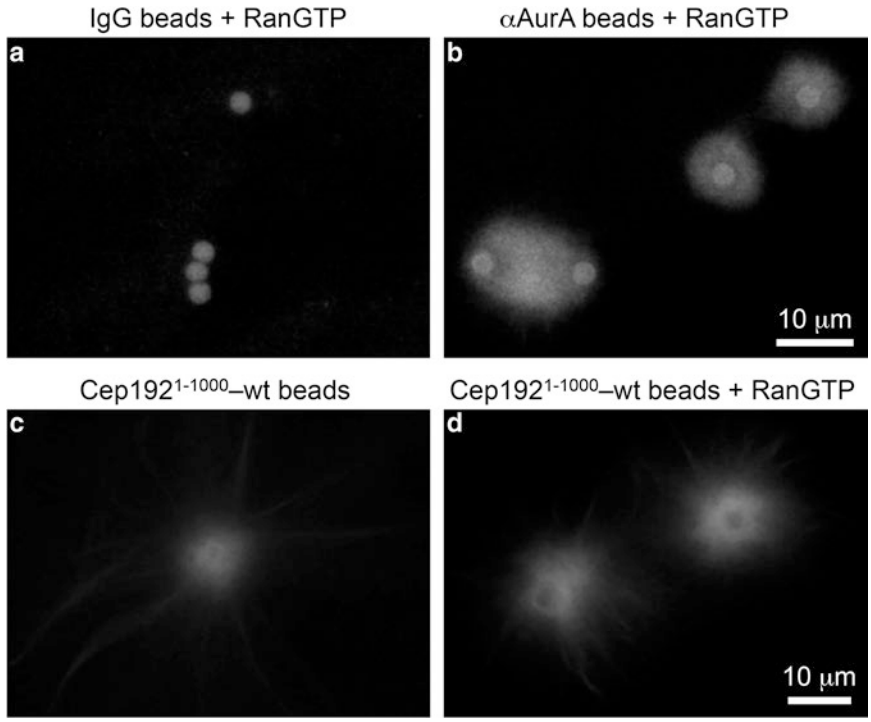

Fig. 3 MT assembly promoted by αAurA beads and Cep192$^{1–1000}$-wt beads in M-phase-arrested *Xenopus* egg extract [10]. The extract was supplemented with rhodamine-labeled tubulin and with beads coated with non-immune IgG (**a**), αAurA (**b**), or Cep192$^{1–1000}$-wt (**c**, **d**) followed by the addition of 8 μM of Ran(Q69L)-GTP (**a**, **b**, **d**) or XB (**c**). Extract aliquots were withdrawn and analyzed by fluorescence microscopy

3.3.3 Analysis of PCM Protein Recruitment Promoted by αAurA Beads

1. Add nocodazole (from a 5 mg/mL stock, Subheading 2.2) to a final concentration of 15 μg/mL to the M-phase-arrested egg extract and mix by pipetting up and down several times.
2. Vortex the stock tube of αAurA beads (Subheading 3.3.1) and transfer $(n+1)$ μL of the bead suspension (where "n" is the total number of extract samples to be analyzed) to a 0.5 mL siliconized Eppendorf tube with 200 μL of XB/1 mg/mL BSA/0.05 % Tween 20. Vortex, spin briefly, and place the tube on the magnet for 1 min. Pipet off the supernatant and wash the beads twice in 200 μL of XB/1 mg/mL BSA/0.05 % Tween 20.
3. Place the tube on the magnet for 1 min, pipet off the supernatant, and add to αAurA beads $(n+1)\times 20$ μL of XB/1 mg/mL BSA/0.05 % Tween 20. Mix the bead suspension by pipetting up and down several times and transfer 20 μL-aliquots to clean 0.5 mL siliconized Eppendorf tubes. Briefly spin the tubes and place them on the magnet.
4. Pipet off all supernatant from the first tube in **step 3** and put the tube on ice. Add 25 μL of the extract from **step 1** (*see* **Note 28**) and Ran(Q69L)-GTP (Subheading 3.1.1) to a final concentration of 8 μM. Mix by pipetting up and down and place the tube

on a rotator (Subheading 2.3) at 4 °C at a low speed. Repeat this step for the other extract samples/tubes in **step 3**.

5. At the desired time interval(s) (20 min–1 h), take the first tube from the rotator, add to it 200 μL of ice-cold XB/0.1 % Tween 20, mix by pipetting up and down, and place the tube on ice. Repeat this step for the other extract samples of the same time point.
6. Spin the tubes briefly and place them on the magnet for 1 min.
7. Pipet off the supernatant from each tube and transfer the tubes to ice.
8. Wash the beads three times with 200 μL of ice-cold XB/0.1 % Tween 20. After the last wash, pipet off the supernatant and add to each tube 30 μL of SDS-PAGE sample buffer. Analyze the samples by SDS-PAGE/Western blotting with antibodies against the proteins of interest.

3.4 Using Cep192 Beads to Model and Study Mitotic Centrosome Formation

The binding sites for AurA, Plk1, γ-TuRC, and the MT polymerase XMAP215 in Cep192 all reside within the N-terminal 1000-amino acid domain of this large (2638 amino acids/290 kDa) protein [10]. Accordingly, beads coated with an oligomerized recombinant form of this domain (Cep192$^{1–1000}$-wt) recapitulate the Cep192-organized AurA-Plk1 kinase cascade and assemble MT asters similar to those formed by αAurA beads in M-phase-arrested egg extract. Moreover, the centrosome-like behavior of Cep192$^{1–1000}$-wt beads does not require the addition of exogenous Ran-GTP to the extract (Fig. 3c, d). However, unlike αAurA beads, Cep192$^{1–1000}$-wt beads do not form bipolar MT spindles, suggesting that this process requires either a region of Cep192 C-terminal to the serine-1000 residue or another AurA-interacting protein(s) [10]. Nevertheless, because Cep192$^{1–1000}$-wt beads represent a defined template, which can be easily manipulated experimentally (e.g., by mutations or other modifications of the bead-bound recombinant Cep192), they can be used as a powerful tool for dissection of the mechanisms of centrosome maturation at steps downstream of AurA activation. Below, we describe a protocol for the preparation of Cep192$^{1–1000}$-wt beads by binding of the corresponding recombinant GST-tagged Cep192 fragment to the beads coated with an anti-GST antibody (Subheading 3.4.1). We then describe how to use Cep192$^{1–1000}$-wt beads as a model to study centrosome-driven MT assembly (Subheading 3.4.2) and PCM protein recruitment (Subheading 3.4.3).

3.4.1 Preparing Cep192$^{1–1000}$ Beads

1. Inoculate 500 mL of LB in a 2 L flask with 10 mL of an overnight culture of *E. coli* expressing the Cep192$^{1–1000}$-wt-GST fusion protein (or its mutant counterpart) [9, 10] and incubate on a shaker at 37 °C. When cell density reaches OD_{600} of ~0.4–0.6 (~1–2 h), add IPTG to a final concentration of 1 mM and continue incubation on a shaker at 25 °C for 4–6 h.

2. Spin down the bacteria by centrifugation at 6000 × *g*, 4 °C for 20 min and resuspend the pellet in 5 mL of ice-cold NETS buffer (*see* **Note 29**). Transfer the bacterial lysate to a 15 mL Falcon tube and place the tube on ice.
3. Weight 5 mg of lysozyme in a 1.5 mL Eppendorf tube, dissolve it in ~200 μL of deionized water, and add to the bacterial lysate. Invert the tube several times and incubate it on ice for 30 min, inverting the tube every 10 min.
4. Sonicate the bacterial lysate on ice, at ~40 % amplitude, 4–6 times (20 s each), with a 1-min interval. Repeat the sonication cycles, if needed, until the lysate is no longer viscous (*see* **Note 30**).
5. Transfer the sample to a 10–15 mL centrifugation tube or to several 1.5 mL Eppendorf tubes and centrifuge at 16,000 × *g*, 4 °C for 30 min.
6. Transfer the supernatant to a new 15 mL Falcon tube and add Triton X-100 (from a 20 % stock, Subheading 2.2) to a final concentration of 4 %. Incubate the tube with a slow rotation for 1 h at 4 °C.
7. Place the tube on ice and snap-freeze 0.5 mL aliquots of the Cep192$^{1–1000}$-wt-GST lysate in liquid nitrogen. Store at –80 °C (*see* **Note 31**).
8. Prepare anti-GST beads according to the protocol described above for αAurA beads (Subheading 3.3.1) except that a rabbit affinity purified anti-GST antibody is used instead of αAurA (*see* **Note 32**).
9. Thaw an aliquot of the Cep192$^{1–1000}$-wt-GST lysate from **step 7** in a water bath at 21 °C and place it on ice.
10. Vortex the stock tube of the anti-GST beads from **step 8** and pipet 10 μL of the bead suspension to a new 0.5 mL siliconized Eppendorf tube with 200 μL of NETN buffer (*see* **Note 33**). Vortex, spin down briefly, and place the tube on the magnet for 1 min. Remove supernatant and wash the beads with 200 μL of NETN buffer. Place the bead suspension on the magnet for 1 min.
11. Pipet off NETN buffer and add 100 μL of the Cep192$^{1–1000}$-wt-GST lysate to the anti-GST beads. Resuspend the beads by pipetting up and down and place the tube on a rotator at 4 °C for 1 h.
12. Spin down the tube briefly and place it on ice. Wash the beads twice with 200 μL NETN buffer/500 mM NaCl and three times with 200 μL of XB/1 mg/mL BSA/0.05 % Tween 20 (Subheading 2.3, all solutions should be kept on ice). Resuspend Cep192$^{1–1000}$-wt beads in 50 μL of XB/1 mg/mL BSA/0.05 % Tween 20 and place the tube with the beads on ice until use (*see* **Note 34**).

3.4.2 Analysis of Cep192$^{1–1000}$ Bead-Induced MT Assembly

1. Thaw one aliquot of the M-phase-arrested extract and add to it rhodamine-labeled tubulin (Subheading 3.1.2, **steps 1** and **2**).
2. Pipet 15 μL of the rhodamine-tubulin-supplemented extract into a new 0.5 mL Eppendorf tube. Add 0.5 μL of Cep192$^{1–1000}$-wt beads (Subheading 3.4.1), mix by pipetting up and down several times, and immediately place the tube on a rotator at room temperature.
3. Every 5–10 min of the incubation, remove the tube from the rotator and take a 0.8 μL aliquot for the analysis by fluorescence microscopy as described in Subheading 3.1.2, **steps 4–6**. Return the tube to the rotator.

3.4.3 Analysis of PCM Protein Recruitment Promoted by Cep192$^{1–1000}$ Beads

1. Add nocodazole (from a 5 mg/mL stock, Subheading 2.2) to a final concentration of 15 μg/mL to the M-phase-arrested extract and mix by pipetting up and down several times.
2. Mix the suspension of Cep192$^{1–1000}$-wt beads (Subheading 3.4.1) and pipet 10 μL aliquots to the new 0.5 mL siliconized Eppendorf tubes (*see* **Note 35**). Spin briefly and place the tubes on the magnet.
3. Pipet off all supernatant from the first tube and transfer the tube to ice. Add 25 μL of the nocodazole-supplemented egg extract to the tube, mix by pipetting up and down several times, and place the tube on the rotator at room temperature. Repeat this step for the other tubes in **step 2**.
4. At the desired time intervals (*see* **Note 35**), take the tube(s) from the rotator and place it on ice. Add 200 μL of ice-cold XB/0.1 % Tween 20 (Subheading 2.3) to the tube and mix by pipetting up and down. Spin down briefly and place the tube on the magnet for 1 min.
5. Pipet off the supernatant and wash the beads three times with 200 μL of ice-cold XB/0.1 % Tween 20. After the last wash, pipet off the supernatant and add 30 μL of SDS-PAGE sample buffer to the tube. Analyze the samples by SDS-PAGE/Western blotting of the proteins of interest.

3.5 Analysis of Centrosome-Driven Spindle MT Assembly in Mammalian Cells

Spindle pole assembly can be assessed in mammalian cells by immunofluorescence of α/β tubulin and spindle pole-specific or enriched proteins, such as Cep192, Cep215, pericentrin, ASPM, γ-tubulin, TPX2, and NuMA. To this end, adherent cells are typically cultured on coverslips, enriched in mitosis, and fixed (usually in methanol or formaldehyde). The coverslips are then analyzed by immunofluorescence by a procedure like that described for the extract-derived spindle MT structures (Subheading 3.2.2, **steps 10–23**). However, the fact that most cultured animal cells have a centrosome makes it difficult to use this experimental system to

study acentriolar spindle formation. Nevertheless, this task can be accomplished by the ablation of centrioles by laser irradiation, microsurgery or by genetic or pharmacological means [29, 30]. To study the MT-nucleating activity of centrosomes, a so-called MT regrowth assay is commonly used. In this assay, cells are first incubated on ice or in the presence of a MT-depolymerizing drug, such as nocodazole, to depolymerize MTs. Then the cells are quickly returned to MT growth-permissive conditions (by adding a warm medium or by washing out the nocodazole) and, after a short (a few seconds to several minutes) incubation at 37 °C, they are fixed and analyzed by immunofluorescence of α/β tubulin to assess MT regrowth from the centrosomes (Fig. 4). Below, we describe a MT regrowth assay optimized for the analysis of centrosome-driven spindle MT assembly in HeLa cells.

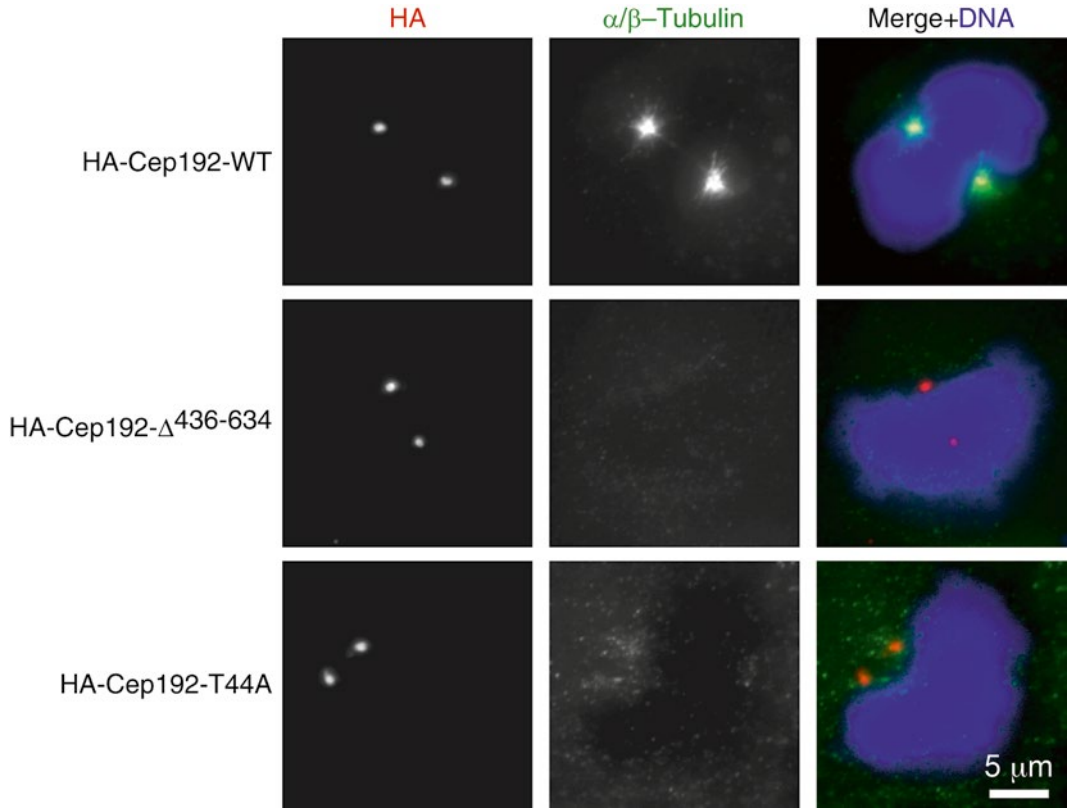

Fig. 4 MT regrowth assay in Cep192-siRNA-treated cells expressing HA-tagged human Cep192-wt or its mutant counterparts lacking the AurA-binding domain (HA-Cep192-$\Delta^{436-634}$) or the N-terminal Plk1-docking threonine 44 (HA-Cep192-T44A). The cells were subjected to a MT regrowth assay followed by immunofluorescence with anti-HA tag and anti-α/β tubulin antibodies. Note the lack of centrosomal MTs and incomplete centrosome separation due to the ablation of the Cep192-organized AurA-Plk1 cascade in cells expressing the mutant forms of Cep192 [10]

1. Using fine-tip forceps, dip each 22 mm × 22 mm #1 coverslip in 95 % ethanol in a beaker, dab off excess fluid against the wall of the beaker, and ignite the ethanol on the coverslip in a flame. After the ethanol has burned off, allow the coverslip to chill and place it in a sterile 35 or 60 mm cell culture dish.
2. Plate HeLa cells in DMEM/10 % FBS in the dishes with coverslips at a density of ~2.5×10^5 or ~5×10^5 cells (per 35 mm or 60 mm dish, respectively). Place the dishes in a humidified incubator at 37 °C, 5 % CO_2 for 24–30 h.
3. Add RO-3306 [31] at a final concentration of 9 μM to each dish, swirl gently, and continue incubating the dish in a humidified incubator at 37 °C, 5 % CO_2 for 18–19 h (*see* **Note 36**).
4. Aspirate the medium and wash the cells three times in ~4 mL or 6 mL (per 35 mm or 60 mm dish, respectively) of DMEM at 37 °C. Add 2 mL or 4 mL (per 35 mm or 60 mm dish, respectively) of DMEM/10 % FBS to the cells and incubate the dishes in a humidified incubator at 37 °C, 5 % CO_2 for 30 min (*see* **Note 37**).
5. Add MG-132 at a final concentration of 20 μM to the cell culture medium (*see* **Note 38**), swirl each dish gently, and place it on an ice water bath for 1 h to depolymerize microtubules.
6. Aspirate the medium and quickly put the dish on the water surface in a water bath at 37 °C. Immediately add 2 mL or 4 mL (per 35 mm or 60 mm dish, respectively) of preheated to 37 °C DMEM/10 %/FBS to the dish and start the timer.
7. After the desired incubation time (20 s–5 min) (*see* **Note 39**), do the following as quickly as possible: aspirate the medium from the dish, wash the cell layer with preheated (37 °C) PBS (Subheading 2.3), aspirate the fluid, and fill the dish with methanol at −20 °C.
8. Cover the dish and store at −20 °C until immunofluorescence analysis.
9. Repeat **steps 6–8** for the other cell culture dishes.
10. Aspirate methanol and wash the cells three times, 5 min each, in TBS-T (Subheading 2.2). Analyze the cells by immunofluorescence of α/β tubulin and of a centriolar/PCM marker, as described in Subheading 3.2.2, **steps 10–23** (use doubled volumes of solutions for washing/staining the coverslips).

4 Notes

1. Recombinant human Ran(Q69L) can be prepared as previously described [27]. Briefly, a cDNA encoding human Ran(Q69L) with N-terminal 6×His-tag and S-tag sequences in a pET29a(+) vector (Novagen) is expressed in BL21 Star (DE3) bacterial

cells (Life Technologies). The protein is purified using Ni-NTA Agarose (Qiagen) according to the manufacturer's instructions. A protocol for the production of an untagged recombinant human Ran(Q69L)-GTP can be found in ref. 26.

2. All solutions described in this chapter should be prepared in deionized water, unless otherwise specified.
3. Although freshly made egg extracts always perform better, all procedures described here (except the experiments in cycling extracts) can be carried out in frozen/thawed extracts. To freeze the extract, add 33 μL of 1.5 M sucrose per each mL of the extract, mix by pipetting up and down, and snap-freeze 50–100 μL aliquots in liquid nitrogen. Store the extract in liquid nitrogen or at −80 °C. Repeated freeze–thaw cycles of egg extracts are not recommended.
4. Egg extracts can be subjected to various experimental treatments, such as immunodepletion and/or addition of inhibitors or recombinant protein(s) of interest, followed by analysis using the methods described in this chapter.
5. An ice bucket filled with water at 21 °C can be used as a water bath in the experiments in egg extracts.
6. Rhodamine-labeled tubulin should preferably be added to the extract prior to the experimental procedures (e.g., immunodepletion or protein additions) to minimize the variability of the fluorescence signals between experimental samples due to pipetting errors. Freeze the remaining aliquot of rhodamine-labeled tubulin in liquid nitrogen and store at −80 °C. Up to three freeze–thaw cycles did not negatively affect the properties of rhodamine-labeled tubulin in the assays described in this chapter.
7. After opening, the ampoule with 16 % formaldehyde can be sealed with Parafilm and stored at room temperature for several weeks.
8. The microscope slides should be clean and devoid of any particles or glass powder. If necessary, clean each slide with Kimwipes soaked in ethanol prior to use.
9. For preparation of cycling egg extracts, we use a modified protocol by Murray [15], wherein eggs are activated by incubation in the presence of the calcium ionophore A23187 (Sigma) [32], instead of by electric pulse.
10. We use a rabbit affinity purified antibody generated against a bacterially expressed 6×His-tagged N-terminal (amino acids 2–199) fragment of *Xenopus* AurA [9]. Beads coated either with this antibody [9, 10] or with a rabbit polyclonal antibody against full-length *Xenopus* AurA [13] nucleate MTs in a centrosome-like fashion in M-phase-arrested *Xenopus* egg

extracts. The purified antibody is dialyzed against PBS/10 % sucrose, concentrated, snap-frozen in aliquots in liquid nitrogen, and stored at −80 °C.

11. The molar concentration of GTP in the solution should be ~100-fold higher than that of Ran(Q69L). M.w. of GTP is 523 Da. M.w. of Ran(Q69L) is ~24.4 kDa.
12. Unless otherwise specified, mixing of egg extracts should be performed by gentle pipetting up and down (5–10 times), avoiding bubbles.
13. To visualize a spindle protein of interest, the extract can also be supplemented with a trace amount of the relevant fluorochrome-labeled antibody, which does not, by itself, affect spindle MT assembly [27, 28] (Fig. 1b).
14. It is critical to minimize the volume of the components added to the egg extract because dilution of the extract negatively affects its performance. The total volume of the additives should not exceed 20 % of the volume of the extract.
15. The speed of formation and the size of the RanGTP-induced MT structures, as well as the proportion between MT asters and spindles, vary depending on the quality of the extract. Extensive experimental procedures, such as multiple rounds of immunodepletion, may inhibit MT aster formation and abolish MT spindle formation.
16. Since the quality of the fixed extract samples deteriorates with time, it is desirable to analyze the samples within a day or two after the preparation.
17. The addition of nocodazole to the extract prevents centrosome-driven MT assembly and improves the visualization of centrosomes by immunofluorescence, in part, by eliminating the background binding of antibodies to MTs. Of note, centrosome maturation precedes and occurs independently of spindle MT assembly.
18. The volume of the extract should be calculated based on the specific purpose of the experiment, particularly depending on whether and at how many time points the samples need to be analyzed by immunofluorescence (Subheading 3.2.2). In most cases, immunofluorescence of centrosomes at two time points (e.g., 5 and 10 min) is sufficient to assess PCM protein recruitment (in the nocodazole-supplemented extracts) and centrosome-driven MT assembly (in the rhodamine-tubulin-supplemented extracts).
19. The formation of centrosomal MT asters begins at ~2 min and peaks at ~10–12 min after the addition of sperm nuclei to the extract. At later time points, centrosomal MT asters may detach from sperm nuclei and the growth and architecture of

centrosomal MTs might be affected by the chromatin/RanGTP-driven pathway of spindle MT assembly.

20. If necessary, several rounds of centrifugation can be performed. After each spin-down, thoroughly rinse the spin-down glass tubes and adaptors with deionized water and dry with Kimwipes. Prolonged (up to 2 h) incubation of the extract samples in spindle dilution buffer did not affect immunofluorescence of centrosomes and MT structures.
21. To prevent wrinkling and bending of the Parafilm, place it on the surface of a bench sprayed with distilled water from a wash bottle. Using Kimwipes, gently press the Parafilm and remove the excess of water from its margins.
22. The speed of cell cycle progression depends on the quality of the extract (some extracts do not cycle) and varies from experiment to experiment. The performance of the extract is very sensitive to prolonged storage on ice, presence of impurities, dilution, and other treatments. Cycling egg extracts need to be freshly prepared and handled with extreme care. Experiments should be well planned and performed immediately after preparation of the extract, without delays.
23. Siliconized/low binding Eppendorf tubes can be used instead of the conventional Eppendorf tubes also in all other experiments described in this chapter.
24. Unless otherwise specified, in all procedures described in this chapter, wash the magnetic beads as follows: (1) add an appropriate washing buffer to the tube containing the beads; (2) resuspend the beads by vortexing for ~20 s; (3) briefly (~5 s) spin the tube with the beads in a microcentrifuge; (4) place the tube on the magnet for ~1 min to retrieve the beads; (5) accurately pipet off the supernatant from the tube on the magnet with a 200 μL or smaller (depending on the volume of the supernatant) pipette tip; (6) immediately transfer the tube with the beads to ice and proceed to the next step of the experiment.
25. Because the amount of αAurA in the binding reaction exceeds the binding capacity of the Dynabeads Protein A, a substantial amount of non-bound antibody may remain in the supernatant and can be used for other applications. The volumes of Dynabeads Protein A and αAurA can be scaled up, if necessary.
26. The purpose of the incubation of αAurA beads with the cross-linker, DMP, is to covalently bind αAurA to Protein A on the bead surface. Because of the extreme sensitivity of DMP to the moisture, the vial should be opened immediately before use and cannot be stored. It is, therefore, practical to purchase small aliquots of the DMP powder.

27. The addition of Ran(Q69L)-GTP to the reaction promotes the αAurA bead-mediated MT growth and spindle formation [13]. However, Ran(Q69L)-GTP also induces the assembly of MT asters and spindles (like those seen in Fig. 1a) independently of αAurA beads. The proportion of such "beadless" MT structures increases with the increase of the Ran(Q69L)-GTP concentration and at later time points.
28. The αAurA bead-to-extract ratio may vary to a certain extent. We recommend αAurA bead-to-extract ratios of ~1:25–1:70 for protein binding experiments and ~1:100–1:200 for the analysis of MT assembly.
29. Because of the poor solubility of the bacterially expressed Cep192$^{1–1000}$-wt, a "semi-denaturing" buffer containing Sarkosyl (NETS buffer) is used to lyse the bacteria. Sarkosyl is then neutralized by incubating the lysate with Triton X-100.
30. To improve sonication efficiency, the sonication can be carried out in a 30 mL round-bottom tube.
31. The Cep192$^{1–1000}$ lysates can be frozen/thawed several times.
32. An anti-GST antibody can be affinity isolated with a GST-resin from an antiserum generated against any GST-tagged protein. The volumes of Dynabeads Protein A and anti-GST antibody can be scaled up, if necessary.
33. The volumes of the anti-GST bead suspension and of the Cep192$^{1–1000}$-wt lysate can be scaled up accordingly if a larger amount of Cep192$^{1–1000}$-wt beads is needed for the experiments.
34. Beads preloaded with mutant forms of Cep192$^{1–1000}$ or with other fragments of Cep192 can be prepared using an analogous procedure. Cep192-coated beads can be stored on ice for several days. The density of the bead-bound Cep192 can be assessed by analyzing an aliquot of the beads by SDS/PAGE/Western blotting.
35. The number of tubes depends on the number of samples and time points to be analyzed. In a typical assay, the PCM protein recruitment is analyzed at two time points, 20 and 40 min after the addition of Cep192$^{1–1000}$ beads to the egg extract.
36. RO-3306 is a Cdk1 inhibitor, which arrests cells at the G2/M transition [31].
37. During the 30 min incubation following the wash-out of RO-3306, the cells exit the G2/M arrest and enter mitosis.
38. MG-132 prevents mitotic exit and, hence, increases the proportion of cells in mitosis.
39. Robust regrowth of MTs from mitotic centrosomes can be seen already after 20–30 s of the incubation.

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