Regulation of Microtubule Assembly and Organization in Mitosis by the AAA+ ATPase Pontin

Daniel Ducat,^{*†} Shin-ichi Kawaguchi,^{*‡} Hongbin Liu,^{§||} John R. Yates, III[§]
and Yixian Zheng^{*†}

^{*}Department of Embryology, Carnegie Institution for Science and Howard Hughes Medical Institute, Baltimore, MD 21210; [†]Department of Biology, Johns Hopkins University, Baltimore, MD 21218; and [§]Department of Cell Biology, The Scripps Research Institute, La Jolla, CA 92037

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To identify novel proteins important for microtubule assembly in mitosis, we have used a centrosome-based complementation assay to enrich for proteins with mitotic functions. An RNA interference (RNAi)-based screen of these proteins allowed us to uncover 13 novel mitotic regulators. We carried out in-depth analyses of one of these proteins, Pontin, which is known to have several functions in interphase, including chromatin remodeling, DNA repair, and transcription. We show that reduction of Pontin by RNAi resulted in defects in spindle assembly in *Drosophila* S2 cells and in several mammalian tissue culture cell lines. Further characterization of Pontin in *Xenopus* egg extracts demonstrates that Pontin interacts with the gamma tubulin ring complex (γ -TuRC). Because depletion of Pontin leads to defects in the assembly and organization of microtubule arrays in egg extracts, our studies suggest that Pontin has a mitosis-specific function in regulating microtubule assembly.

INTRODUCTION

Timely progression through the mitotic phase of the cell cycle and the assembly of a bipolar mitotic spindle is a complex process involving cooperation of a variety of cellular components and inputs from multiple cellular compartments. In animal cells, both chromosomes and centrosomes participate in spindle assembly. In addition to providing the kinetochores for assembly of the kinetochore fibers of the spindle (reviewed in O'Connell and Khodjakov, 2007), chromosomes help to organize the mitotic spindle by providing a spatial RanGTP gradient that guides spindle assembly toward the chromatin (Kalab *et al.*, 2002, 2006; Li *et al.*, 2003; Li and Zheng, 2004). Centrosomes, in contrast, facilitate spindle assembly by providing sites for microtubule nucleation and organization. Centrosomes also help to concentrate various spindle assembly factors at the spindle poles during mitosis.

To better understand the full complement of factors involved in spindle assembly and chromosome segregation, both proteomic and genomic-based approaches have been used to compile an inventory of proteins that regulate mitosis (Kittler *et al.*, 2004; Liska *et al.*, 2004; Skop *et al.*, 2004; Sauer *et al.*, 2005; Goshima *et al.*, 2007; Kittler *et al.*, 2007). In addition to expected spindle assembly factors and uncharacterized proteins, these efforts have identified a large number of proteins with established functions in interphase.

Because many of these proteins have known roles in regulating interphase membrane trafficking, gene transcription, or protein translation, their involvement in mitosis could be indirect. However, evidence is accumulating to suggest that some proteins with well-established interphase roles, including nuclear or membrane trafficking functions, can also directly regulate mitosis (Carazo-Salas *et al.*, 1999; Kalab *et al.*, 1999; Ohba *et al.*, 1999; Wilde and Zheng, 1999; Gruss *et al.*, 2001; Nachury *et al.*, 2001; Wiese *et al.*, 2001; Cao *et al.*, 2003; Ems-McClung *et al.*, 2004; Royle *et al.*, 2005; Vong *et al.*, 2005; Tsai *et al.*, 2006).

For example, the type V intermediate filament protein lamin B functions as a structural component of the interphase nuclear lamina, and it is required for spindle assembly in mitosis (Tsai *et al.*, 2006). The endocytic protein clathrin is localized to the mitotic spindle where it regulates chromosome alignment (Royle *et al.*, 2005). Several transcription factors and components of chromatin remodeling complexes have been shown to localize to mitotic centrosomes, spindle poles, and/or throughout the mitotic spindle, where some seem to directly assist spindle assembly (Xue *et al.*, 2000; Kaplan *et al.*, 2004; Xu *et al.*, 2005; Parvin and Sankaran, 2006; Huang *et al.*, 2007; Sillibourne *et al.*, 2007). In addition, multiple interactions have been observed between chromatin remodeling complexes and kinetochore proteins by yeast two-hybrid analyses (Wong *et al.*, 2007). In one recent study, members of the nucleosome remodeling deacetylase (NuRD) complex, including chromodomain helicase DNA-binding proteins (CHD3 and CHD4), were shown to interact with pericentrin, an integral centrosomal component (Sillibourne *et al.*, 2007). CHD3 and CHD4 were found to localize to centrosomes both in interphase and mitosis. Furthermore, reduction of CHD3 by RNA interference (RNAi) resulted in mitotic defects, including aberrant spindle assembly and chromosome missegregation. Because reduction of CHD3 displaced pericentrin and γ -tubulin from centrosomes, this

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Present addresses: [†]Temasek Life Sciences Laboratory, Singapore 117604, Singapore; ^{||}Protein Analytical Chemistry, Genentech, South San Francisco, CA 94080.

Address correspondence to: Daniel Ducat (ducat@ciwemb.edu) or Yixian Zheng (zheng@ciwemb.edu).

chromosome remodeling protein seems to regulate mitotic centrosome integrity and function (Sillibourne *et al.*, 2007).

Localization studies have implicated additional chromatin remodeling complex components, including Pontin and Reptin, in mitosis (Gartner *et al.*, 2003; Sigala *et al.*, 2005). Pontin (also called Pontin52, TIP49, TIP49a, RuvBL1, Rvb1, TAP54 α , TIH1p, ECP-54, p55, or hNMP 238) and Reptin (also called TIP48, TIP49b, RuvBL2, Rvb2, TAP54 β , TIH2p, ECP-51, or p50) are closely related members of the AAA+ family of ATPases found to regulate a variety of proteins. Pontin and Reptin interact with one another, and they can assemble into stacked hexamers surrounding a central channel (Puri *et al.*, 2007). Both proteins have been shown to regulate chromatin state and gene expression through their association with several chromatin remodeling complexes and transcription factors (reviewed in Gallant, 2007). In mitosis, both Pontin and Reptin localize to mitotic spindles and spindle poles, whereas Reptin alone localizes to midbodies (Gartner *et al.*, 2003; Sigala *et al.*, 2005). Because glutathione transferase (GST)-tagged Pontin could pull-down α -tubulin and γ -tubulin in human U937 cells (Gartner *et al.*, 2003), it was suggested that Pontin and/or Reptin, like the NuRD components, might have direct functions in mitosis.

Using a proteome-based approach, we have identified candidate mitotic regulators including Pontin and Reptin. We used an RNAi-based, phenotypic screen to identify the mitotic regulators among these candidates. Here, we report the screen results and further characterization of the mitotic function of one of the identified proteins, Pontin, by using both tissue culture cells and *Xenopus* egg extracts.

MATERIALS AND METHODS

Buffers and Reagents

HEPES buffer (HB): 50 mM HEPES, pH 8.0, 1 mM MgCl₂, 1 mM EGTA, and 1 mM β -mercaptoethanol. HB100K and HB250K, HB containing 100 or 250 mM KCl, respectively. HB100Na: HB containing 100 mM NaCl. *Drosophila* embryo homogenization buffer: HB100Na with 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM benzamide-HCl, 10 μ g/ml aprotinin (GE Healthcare, Chalfont St. Giles, United Kingdom), 10 μ g/ml leupeptin (Sigma-Aldrich, St. Louis, MO), and 10 μ g/ml pepstatin A (Calbiochem, San Diego, CA). MudPIT digestion buffer: 100 mM ammonium bicarbonate, pH 8.5, 2 M urea, and 1 mM CaCl₂. PHEM buffer: 60 mM piperazine-N,N'-bis(2-ethanesulfonic acid), pH 6.8, 25 mM HEPES, 10 mM EGTA, and 4 mM MgCl₂. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, and 2 mM KH₂PO₄ adjusted to pH 7.4. Purification buffer for GST-tagged protein: 50 mM HEPES, pH 8.0, 1 mM EGTA, 1 mM MgCl₂, 100 mM NaCl, 1 mM PMSF, 0.1% Triton X-100, and 1 mg/ml lysozyme. Protease inhibitor stock: 10 mg/ml each of leupeptin (Millipore Bioscience Research Reagents, Temecula, CA), pepstatin A (Calbiochem), and chymostatin (Millipore Bioscience Research Reagents) in dimethyl sulfoxide. GST buffer A: 50 mM HEPES, pH 8.0, 100 mM NaCl, 100 mM KCl, 1 mM EGTA, and 1 mM MgCl₂. GST buffer B: GST buffer A containing 10 mM glutathione. His buffer A: 50 mM Na-phosphate, pH 8.0, 300 mM NaCl, and 1 mM PMSF. Lysis buffer for purification of His₆-tagged proteins: His buffer A supplemented with 0.1% Triton X-100 and 1 mg/ml lysozyme. Buffer R: 50 mM Na phosphate, pH 8.0, 100 mM NaCl, 1 mM PMSF, 1 mM dithiothreitol, 1 mM EDTA. XB: 10 mM HEPES, pH 7.7, 50 mM sucrose, 100 mM KCl, 1 mM MgCl₂, 0.1 mM CaCl₂, and 5 mM EGTA.

Biochemical Enrichment of Proteins Required for Microtubule Nucleation from Centrosomes

Drosophila embryos between 0 and 2 h old were dechorionated and homogenized to make crude embryo extract as described previously (Gunawardane *et al.*, 2000). Clarified extracts were prepared by centrifugation at 50,000 rpm in a SW55 rotor for 20 min at 4°C in the presence of 100 μ M nocodazole. *Drosophila* centrosomes were isolated from 0- to 3.5-h *Drosophila* embryos, incubated with 2 M KI, and allowed to bind to glass coverslips, as described previously (Moritz *et al.*, 1998). Fractionation of the clarified embryo extracts were carried out as described previously (Kawaguchi and Zheng, 2004). Briefly, the extracts were first precipitated by the addition of Polyethylene glycol (PEG) 8000 to a final concentration of 3% and incubated on ice for 20 min. The precipitated proteins, which included ~90% of the total γ -tubulin ring complex (γ TuRC), were resuspended in HB100K supplemented with 0.1

mM guanosine triphosphate and 0.05% NP-40 and clarified by centrifugation. The supernatant was loaded onto 5–40% sucrose gradients prepared in HB100K by using previously described methods (Oegema *et al.*, 1999; Gunawardane *et al.*, 2001). Gradients were centrifuged in a SW55 rotor at 50,000 rpm for 4 h at 4°C. The gradient fractions were tested for centrosome-complementing activity as described previously (Kawaguchi and Zheng, 2004). Briefly, 60 μ l of either total PEG-precipitated proteins or 60 μ l of individual sucrose fractions were incubated with centrosomes that had been affixed to coverslips and pretreated with 2 M KI. After 15-min incubation at 30°C, soluble proteins were washed away, and purified tubulin and rhodamine-labeled tubulin were added and incubated for 10 min at 30°C. Centrosome complementation activity was assayed by the number of microtubule asters nucleated from reconstituted centrosomes. The fractions with peak centrosome-complementation activity (which contained γ TuRC) were combined, filtered, and further purified using a Mono S column (HR 5/5; GE Healthcare) and a linear NaCl gradient (0.1–0.5 M) in HB. The fraction displaying the peak centrosome-complementing activity was analyzed by mass spectrometry using Multidimensional Protein Identification Technology (MudPIT).

Enrichment of centrosome-complementing activity was observed in peak fractions obtained from both sucrose gradient sedimentation and Mono S affinity chromatography. Enrichment of complementing activity relative to protein concentration was estimated as >7.5-fold for peak sucrose gradient fractions (~75% activity of total PEG-precipitated load, after a >10-fold dilution on sucrose gradient). Enrichment of complementing activity of the peak Mono S fraction was ~4.5-fold compared with the activity of the pooled sucrose gradient fractions (see Kawaguchi and Zheng, 2004; Figure 4).

MudPIT

Precipitated *Drosophila* centrosome-complementing proteins were dissolved in digestion buffer, digested with trypsin, and analyzed by liquid chromatography/liquid chromatography tandem mass spectrometry (LC/LC/MS/MS) according to published protocols (Washburn *et al.*, 2001). Approximately 200 μ g of protein was used for a 12-step LC/LC/MS/MS experiment. Tandem mass spectra were analyzed by SEQUEST using the aa_gadfly.dros. RELEASE2 database (downloaded from <http://www.fruitfly.org>; release date October 2000). The SeQUEST outputs were then analyzed by DTASelect (Tabb *et al.*, 2002). The DTASelect filter settings were as follows: XCorr: +1 ions 1.8, +2 ions 2.5, +3 ions 3.5; delta CN: 0.08; a protein was identified when two or more of its peptides were sequenced. Proteins with four or more unique peptides that passed the DTASelect filter were considered real hits. Proteins with two to three peptides that passed the DTASelect filter were further manually validated.

Double-stranded RNA Synthesis

We used the *Drosophila* RNAi Library 1.0 from Open Biosystems (Huntsville, AL; <http://www.openbiosystems.com>) to generate double-stranded (dsRNAs) for our RNAi screen using S2 cells (Foley and O'Farrell, 2004). dsRNAs were synthesized using MEGAscript T7 In Vitro Transcription kits (Ambion, Austin, TX) and recovered by phenol:chloroform extraction and isopropanol precipitation as described by the manufacturer. The dsRNA was annealed and analyzed by UV absorbance for quantification (BioSpec-1601; Shimadzu, Kyoto, Japan) and by electrophoresis in 1.5% agarose gels to ensure that the RNA migrated as a single band. Control dsRNA targeting the enhanced green fluorescent protein (EGFP) gene was similarly generated using polymerase chain reaction (PCR) primers (forward-TAATACGACTCACTATAGGGACTTGCACCACCGCAAGCTG and reverse-TAATACGACTCACTATAGGACTCCAGCTTGTCCTCCAG) to clone an ~300-bp fragment of EGFP (template pEGFP-N3; BD Biosciences, San Jose, CA) DNA with flanking T7 promoters for the production of dsRNA.

Cell Culture, RNAi Interference, and Cell Imaging

Drosophila Schneider cell line 2 (S2 cells) were cultured in Schneider's *Drosophila* medium supplemented with 15% fetal bovine serum (Invitrogen) and transfected according to established methods (Echalier, 1997; Sohail, 2005). Then, 20 μ g of dsRNA and 20 μ l of Transfast (Promega, Madison, WI) were used to transfect each well in six-well plates. S2 cells were resuspended 4 d after transfection, replated on poly-L-lysine-coated glass coverslips for 1 h, and then fixed in 4% paraformaldehyde in PHEM buffer for 15 min. After fixation, S2 cells were permeabilized in PBS + 0.1% Triton X-100 for 1 min, blocked in 4% bovine serum albumin (BSA) in PBS for 1 h, and then immunostained simultaneously with rabbit anti-CP309 (α C2; Kawaguchi and Zheng, 2004) and mouse anti- α -tubulin (DM1 α ; Sigma-Aldrich) antibodies for 2 h. After washing, coverslips were incubated with Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 594 anti-mouse secondary antibodies (Invitrogen, Carlsbad, CA) for 1 h and then stained with 1 μ g/ml 4,6-diamidino-2-phenylindole (DAPI) for 5 min to visualize DNA. HeLa and U2OS cells were cultured in DMEM supplemented with 10% fetal bovine serum (Invitrogen). MCF10A cells were cultured in DMEM/Ham's F-12 medium (Sigma-Aldrich) supplemented with 5% horse serum (Sigma-Aldrich), 20 ng/ml epidermal growth factor (PeproTech, Rocky Hill, NJ), 0.5 mg/ml hydrocortisone (Sigma-Aldrich), 100 ng/ml cholera toxin (Sigma-Aldrich) and 10 μ g/ml insulin. Cells were transfected in log phase with 20 pmol of STEALTH small inter-

fering RNA (siRNA) oligonucleotides (oligos; Invitrogen) corresponding to Pontin (Invitrogen: oligo#1 UGUGACUUCACCUCAUAAACUCC, oligo#2 UUGUACCACCUUUAUUAUCUCC, oligo#3 AUUCCUGUGGAGUAUACAGCAUGG); Reptin (oligo#1 UUUUGUUGUGGUGUAACG-GUUGCC, oligo#2 UUGCUGUGCAUCAAUCUGGAUCUC); or MidG2 Control STEALTH RNAi (Invitrogen) using 4 μ l of Oligofectamine (Invitrogen) in 400 μ l of Opti-MEM plus 1 ml of antibiotic-free DMEM overnight, as per instructions of the manufacturer. Cells were fixed 48–72 h after transfection in -20°C methanol. After fixation, HeLa, U2OS, and MCF10A cells were double stained using the following combinations of antibodies: mouse anti- α -tubulin (DM1 α ; Sigma-Aldrich) and rabbit anti-pericentrin (ab4448; Abcam, Cambridge, MA); rabbit anti- α -tubulin (ab15246; Abcam) and mouse anti- γ -tubulin (GTU-88; Sigma-Aldrich); mouse anti- α -tubulin and rabbit peptide anti-Pontin (this study); or mouse anti- α -tubulin and rabbit anti-Xgrip109 (Martin *et al.*, 1998).

All S2 cell screening was performed on a Nikon Eclipse E800 microscope by using a 100 $\times$ objective (numerical aperture 1.4). Representative images were acquired on a Leica SP5 confocal microscope at 1024 $\times$ 1024 resolution with two-line averaging. Fluorescence images for human cell lines and microtubule structures assembled in *Xenopus* egg extracts were acquired on a Nikon Eclipse E800 microscope equipped with a Hamamatsu Orca2 charge-coupled device (CCD) camera. Confocal z-stacks were acquired on a Yokogawa CSU10 spinning disk confocal equipped with a Hamamatsu C9100-02 EMCCD camera or a Leica SP5 confocal microscope. Maximum projections were generated from acquired stacks. These images were used to measure γ -tubulin or TUBGCP3 staining intensity. The intensity ratio of γ -tubulin (spindle pole/cytoplasmic) or TUBGCP3 was calculated by integrating the average intensity of a $1 \times 1\text{-}\mu\text{m}$ region centered on the spindle pole relative to a representative region in the cytoplasm. Live imaging analysis was performed using a 20 $\times$ lens on a Nikon TE2000 inverted microscope with a Photometrics Cool Snap HQ CCD camera and equipped with a Live Cell incubation chamber (Pathology Devices, Westminster, MD). Fluorescence and brightfield images were acquired every 3 min for a total of ~ 72 h, beginning 24–28 h after transfection. We monitored the progression of cells throughout the length of the experiment, defining cell death by the onset of membrane blebbing and DNA compaction and/or fragmentation. Mitotic death was scored when cells entered mitosis (as viewed by cell rounding, nuclear envelope breakdown, and chromosome compaction), but we did not fully align or segregate DNA before dying. Death in cytokinesis was defined as simultaneous death of connected daughter cells between 0 and 5 h after the onset of anaphase.

Statistical Analysis for RNAi Screen

Data were collected blindly for each RNAi experiment in S2 cells and recorded using a supplemental spreadsheet accompanying another recent screen (Bettencourt-Dias *et al.*, 2004). Individual mitotic cells were classified according to mitotic phase and observed defects were assigned to one or more of 18 potential mitotic phenotypic abnormalities (Supplemental Figure 1). To compare dsRNA treatments to one another, we generated phenotypic scores (PS) for each dsRNA treatment in categories for centrosome, spindle, chromosome, and cytokinetic defects by using similar methods to those described previously (Bettencourt-Dias *et al.*, 2004). We also assayed for prometaphase arrest by comparing the index of cells in prophase through metaphase (ProM). PS represents the ratio of observed mitotic defects in a given category for a candidate gene relative to the defects observed in control dsRNA treatments performed on the same day. $\text{PS} = \log((100 - x)/(100 - c))$, where x is percentage of observed phenotype in a given sample and c is average percentage of same phenotype observed in control samples performed on the same day.

Control experiments were compared with other controls from both same-day and neighboring-day experiments to measure distribution of control PSs and generate confidence intervals (CI) that account for experimental variation and age of the cell culture (Bettencourt-Dias *et al.*, 2004). Specifically, the controls (n , generally 3) for a same-day experiment were sampled, and then $n + 1$ controls were randomly sampled with replacement from neighboring-day experiments. Control PSs were generated by allocating one control data point to the numerator, averaging the remaining controls in the denominator, and taking the log of the ratio (see PS equation above). This process was repeated for each control RNAi experiment performed, generating a bank of 105 control PSs. Novel genes were classified as mitotically relevant only if they had a PS above the 95% CI for control scores in two independent experiments. Phenotypic data associated with spindle, chromosome, and cytokinetic defects (Supplemental Figure 1A) were grouped to generate a categorical PS and minimize false positive results. For experiments with significantly higher PS scores relative to controls, we generally observed increased defects in only one or two subcategories. Therefore, we report the predominant phenotypes contributing toward the overall increase in the PS in each category (Table 1). Due to the mutually exclusive nature of centrosome number low (CNL) and centrosome number high (CNH) phenotypes, PSs for each of the three centrosome-associated defects (CNL, CNH, and centrosome position defect [CPD]) were analyzed independently.

Plasmids, Protein Purification, and Antibodies

We cloned full-length *Xenopus* Pontin and Reptin from IMAGE clones (xPontin: #6631335, xReptin: #5536887; American Type Culture Collection, Manassas, VA) into the BamHI and XhoI sites of pGEX 6P-2 and pET30a to generate constructs for the production of GST-tagged and His₆-tagged recombinant proteins, respectively. Primers used were forward-TAGGGCGGATCCAT-GAAATCGAGGAGGTG, reverse-TAGGGCCTCGAGTATTTTCATGAAC-TTTCTTGCAC and forward-TAGGGCGGATCCATGGCAACCAATGGCG-GCAAC, reverse-TAGGGCCTCGAGTCAGGATGTGTCCATGGTG for Pontin and Reptin, respectively. Proteins were individually expressed and purified from *Escherichia coli* BL21plysS-DE3 competent cells after 4-h isopropyl β -D-thiogalactoside induction at 37°C for antibody production, and purified together for rescue experiments in *Xenopus* egg extracts.

To purify GST-tagged Pontin or Reptin, cells were suspended and lysed in GST buffer A supplemented with 1:1000 protease inhibitor stock and by two sequential centrifugations at 15,000 rpm in an SS-34 rotor (Sorvall, Newton, CT) for 10 min. The supernatant was filtered and applied to a HiTrap GST-Trap HP (GE Healthcare) column. The column was washed with 10 column volumes GST buffer A, and GST buffer B was used to elute the protein from the column. Cleared lysates for cells expressing His₆-tagged Pontin and/or Reptin were prepared similarly to GST-tagged proteins, and they were applied to a HiTrap His-Trap HP column (GE Healthcare). The column was washed with His buffer A containing 30 mM imidazole, and the protein was eluted with a gradient (1–500 mM) of imidazole. Peak fractions were dialyzed 4 h in buffer R and loaded onto a Mono Q 5/50 GL anion exchange column (GE Healthcare), washed with buffer R, and eluted using a 0.1–1 M NaCl gradient. His₆-tagged proteins to be used in *Xenopus* egg extract experiments were exchanged into XB buffer using PD-10 desalting columns (GE Healthcare). All protein purification steps were performed on an AKTA fast-performance liquid chromatography system (GE Healthcare).

Polyclonal antibodies against Pontin and Reptin were generated in rabbits using GST-tagged *Xenopus* Pontin or Reptin recombinant full-length proteins as antigens, and the sera were affinity purified using His₆-tagged Pontin or Reptin, respectively. A second antibody recognizing Pontin was raised against the C-terminal peptide CKSSAKILAEQQEK of *Xenopus* Pontin and affinity purified using the same peptide. Because Pontin and Reptin are highly conserved proteins, the *Xenopus* Pontin and Reptin antibodies recognized their respective proteins in both *Xenopus* egg extracts and human tissue culture cells.

Western analysis for Pontin and Reptin was typically performed using previously described mouse monoclonal antibodies against Pontin (Weiske and Huber, 2005) and Reptin (Weiske and Huber, 2005). Similar results were obtained using the Pontin/Reptin antibodies generated during the course of this investigation. Other antibodies used were as follows: rabbit anti-Xgrip109 (Martin *et al.*, 1998), rabbit anti-Xgrip210 (Zhang *et al.*, 2000), mouse anti- γ -tubulin (GTU-88; Sigma-Aldrich), mouse anti- α -tubulin (DM1 α ; Sigma-Aldrich), and horseradish peroxidase-conjugated secondary antibodies (GE Healthcare).

Manipulation of *Xenopus* Egg Extracts

Cytostatic factor (CSF)-arrested *Xenopus* egg extracts and AurA beads were prepared as described previously (Murray, 1991; Tsai and Zheng, 2005). Ran-induced spindle assembly was performed as described previously (Wilde and Zheng, 1999). To induce spindle assembly using AurA-beads and RanL43E, AurA-beads were first incubated in the egg extracts for 30–45 min at 4°C . RanL43E and rhodamine-labeled tubulin were then added to the egg extracts to induce spindle assembly (Tsai *et al.*, 2006). To immunodeplete Pontin or Reptin, polyclonal antibodies were bound to 100 μ l of protein A-coupled magnetic bead suspension ($\sim 10^9$ beads/ml; Dynal, Biotech, Lake Success, NY) and washed in XB buffer before addition to 100 μ l of egg extracts. An equivalent amount of immunoglobulin (Ig)G from nonimmunized rabbits (The Jackson Laboratory, Bar Harbor, ME) was used for mock depletion controls. Antibody-coated beads and egg extracts suspensions were rotated for 1 h at 4°C before beads were precipitated using a magnet as per manufacturer's instructions ($2 \times 15\text{-min}$ magnet exposure). To achieve $\sim 90\%$ depletion of Pontin, a second round of immunodepletion was performed.

Xenopus γ TuRC was purified using peptide antibody affinity chromatography. Briefly, 3 ml of egg extract was incubated with 60 μ g of antibody made using the XenC peptide (CAATRPDIYSWGTQDK), which corresponds to the last 16 amino acids of *Xenopus* γ -tubulin (Zheng *et al.*, 1995), for 1 h with rotation at 4°C . The immune-complex was recovered using Affi-Prep protein A support beads (Bio-Rad, Hercules, CA). After washing the beads three times with HB100K, γ TuRC was eluted using HB250K containing 1.5 mg/ml XenC peptide, 1:1000 dilution of protease inhibitor stock, and 1 mM GTP. Endogenous levels of γ TuRC, Pontin, and Reptin were recovered by adding purified γ TuRC, Pontin, and Reptin to the egg extracts depleted with Pontin antibodies. We used two strategies to purify recombinant Pontin and Reptin. One strategy was to express *Xenopus* His₆-tagged Pontin and Reptin separately and purify them together. The other was to coexpress human Reptin with human Pontin-His₆ from a bicistronic vector (Puri *et al.*, 2007) followed by purification. Both preparations yielded a complex of Pontin and

Table 1. Mitotic regulators identified by RNAi screen in S2 cells

Expected hits	CG no.:	Defective phenotypes
γ -Tubulin at 23C ^b	CG3157	Centrosome (99%, CNL); Spindle (99%, SMO); Chromosome (99%, CRAD); ProM 95% ^a
Dynein light intermediate chain	CG1938	Sindle (95%, SSP); ProM 99% ^a
Grip84 ^b	CG3917	Spindle (99%, AS); Chromosome (99%, CRAD); Centrosome (95%, CPD)
Grip71 ^b	CG10346	Centrosome (95%, CNL); Chromosome (95%, CRAD, CRCD)
Glued ^b	CG9206	Chromosome (99%, CRAD, CRNH); Spindle (95%, SSP); ProM 95% ^a
Polo kinase ^b	CG12306	Centrosome (99%, CNL); Chromosome (99%, CRAD); Cytokinesis (99%, UD); Spindle (95%, SMO)
Minispindles ^b	CG5000	Spindle (99%, AS); Centrosome (99%, CNL); ProM 99% ^a
SMC2 ^b	CG10212	Chromosome (99%, CRCD & CRAD); ProM ^a
Topoisomerase 2 ^b	CG10223	Chromosome (99%, CRAD); Spindle (99%, AS); ProM 99% ^a
Klp3A ^b	CG8590	Spindle (95%, SSP); Centrosome (95%, CPD)
Klp61F ^b	CG9191	Centrosome (99%, CNL); Chromosome (99%, CRAD), Spindle (99%, SMO)
Lethal (1) G0022 (TCP-1- ζ) ^b	CG8231	Spindle (95%, SMO)
Importin β	CG2637	Chromosome (99%, CRAD); Cytokinesis (99%, CSD); Spindle (95%, AS)
Fizzy	CG4274	Spindle (99%, SSP); Chromosome (99%, CRAD, CRCD); ProM 95% ^a
Actin-related protein 87C	CG6174	Centrosome (95%, CPD); ProM 95% ^a
Cdc16	CG6759	Chromosome (99%, CRAD); ProM 95% ^a
Cdc27	CG8610	Chromosome (99%, CRAD); Centrosome (95%, CPD); Spindle (95%, AS); ProM 95% ^a
Shattered	CG9198	Spindle (95%, AS)
APC10	CG11419	Chromosome (95%, CRAD); ProM 95% ^a
Dynein light chain 90F	CG12363	Chromosome (95%, CRAD), Spindle (95%, AS)
SkpA	CG16983	Centrosome (95%, CNH)
Casein kinase II- α subunit ^b	CG17520	Centrosome (95%, CPD)
U2A ^b	CG1406	Cytokinesis (95%, CSD); Centrosome (95%, CPD)
SmD3 ^b	CG8427	Cytokinesis (99%, CSD); Spindle (95%, AS); Centrosome (95%, CPD)
Noisette ^b	CG2925	Cytokinesis (95%, CSD)
snRNP D2 ^b	CG1249	Cytokinesis (95%, CSD)
Splicing factor 3a, subunit 1 ^b	CG16941	Centrosome (95%, CNL)
eIF-4G ^b	CG10811	Spindle (99%, AS, SMO); ProM 99% ^a
eIF s4 ^b	CG8636	Centrosome (99%, CPD)
eIF 4E ^b	CG4035	Spindle (95%, AS)
eIF 4A ^b	CG9075	Spindle (95%, AS, SSP); ProM
BAP55 ^b	CG6546	Spindle (99%, SSP); Centrosome (99%, CNL; 95%, CPD)
Rpl15 ^b	CG3284	Centrosome (99%, CNL)
Novel hits		
Pontin	CG4003	Spindle (99%, SSP, AS); Centrosome (CNL 99%, CPD 95%)
Cheerio	CG3937	Chromosome (95%, CRCD); Spindle (95%, AS)
Lark	CG8597	Chromosome Defect (99%, CRAD, CRCD); Spindle (99%, AS, SSP)
Bicaudal C	CG4824	Chromosome (95%, CRCD)
Ribosomal protein S3	CG6779	Centrosome (95%, CNL)
Mediator complex subunit 22	CG3034	Spindle (95%, SSP)
B52	CG10851	Spindle (95%, AS, SMO)
Hrp59	CG9373	Chromosome (99%, CRAD); Spindle (99%, AS, SMO)
CG12749	CG12749	Cytokinesis (99%, CSD)
CG3436	CG3436	Spindle (95%, SMP)
CG5931	CG5931	Centrosome (95%, CNL)
CG7671 (nucleoporin 43)	CG7671	Chromosome (95%, CRCD, CRAD)
CG11414	CG11414	Cytokinesis (95%, CSD)

Mitotic regulators identified by RNAi-mediated phenotypic screening using *Drosophila* S2 cells. A total of forty-six genes were identified as essential for mitosis. The 'expected hits' list is comprised of genes that were previously characterized as mitotic regulators. The 'novel hits' list contains 13 novel mitotic regulators identified in this screen. Defective phenotypes reported reflect the PS that are above the 95% or 99% confidence interval for each of the four major categories (chromosome, spindle, centrosome, and cytokinesis) analyzed. The sub-categories listed in parentheses indicate the major contributing defects. Descriptions of observed phenotypes by sub-category: CNL: centrosome number low; CNH: centrosome number high; CPD: centrosome position defect; AS: abnormal spindle; SMO: monopolar spindle; SSP: spindle pole splayed; CRAD: chromosome alignment defect; CRCD: chromosome condensation defect; CRNH: chromosome number high; UD: uneven DNA; CSD: central spindle defect (see Supplemental Fig. 1 for a list of all mitotic phenotypes scored and examples).

^a ProM, high percentages of cells in prometaphase. Phenotypic score confidence level (95% or 99%) was indicated in each category.

^b Mitotic regulators identified in previous RNAi screens within S2 cells (Bettencourt-Dias *et al.*, 2004; Goshima *et al.*, 2007).

Reptin that comigrated on sucrose gradients and both were able to rescue the microtubule assembly defects in Pontin-depleted egg extracts.

Sucrose gradients for the fractionation of *Xenopus* egg extracts were poured as step gradients (five 400- μ l steps) and incubated at 4°C overnight to generate continuous gradients. The gradients were formed from 15 to

40% sucrose (Ultrapure; Sigma-Aldrich) in XB buffer. Then, 50 μ l of clarified *Xenopus* egg extracts was loaded onto each gradient and centrifuged at 4°C at 50,000 rpm for 2.5 h in a TLS55 rotor (Beckman Coulter, Fullerton, CA). The gradients were fractionated by hand from the top into 19 110- μ l fractions. Protein standards (1 mg/ml) were loaded on separate

gradients prepared and centrifuged in the same manner as described above.

RESULTS

Biochemical Enrichment of Proteins Required for Microtubule Nucleation from Centrosomes

Two genome-scale RNAi-based screens have recently been used to identify genes required for mitosis (Kittler *et al.*, 2004; Goshima *et al.*, 2007; Kittler *et al.*, 2007). By necessity, these types of screens require high-content image capturing at low magnifications. Therefore, although several novel genes have been identified, this approach could miss genes with relatively subtle mitotic phenotypes. To complement large-scale screening efforts, we chose to biochemically enrich for mitotically relevant factors and identify candidate proteins by using proteomic analyses. This biochemical approach would yield a shorter list of proteins that is permissive to a manual screen at higher resolution, which could lead to identification of mitotic regulators missed by large-scale screening.

To biochemically enrich for mitotically relevant factors, we turned to the early embryos of *Drosophila melanogaster* that are known to cycle rapidly between mitosis and interphase. Judging by their robust microtubule-nucleating activity in vitro, centrosomes isolated from the early embryos should contain a high proportion of mitotic centrosomes. Previous research has shown that treatment of isolated *Drosophila* centrosomes with chaotropic agents, such as KI, abolishes their ability to nucleate and organize microtubules in solutions of purified tubulin (Moritz *et al.*, 1998; Schnackenberg *et al.*, 1998, 2000). The microtubule-nucleating activity of the salt-stripped centrosomes can be restored by incubation with cytosolic extracts (Moritz *et al.*, 1998; Schnackenberg *et al.*, 1998; Kawaguchi and Zheng, 2004). Because both the centrosomes and extracts are from early *Drosophila* embryos and as mitotic centrosomes are known to tether mitotic regulators, we reasoned that this centrosome-complementation assay could be used to biochemically enrich for mitotic regulators.

Among the factors known to support microtubule nucleation from salt-stripped centrosomes, gamma tubulin ring complex (γ TuRC) (Zheng *et al.*, 1995) is essential but not alone sufficient (Moritz *et al.*, 1998; Schnackenberg *et al.*, 1998; Kawaguchi and Zheng, 2004). To enrich for all proteins required for complementation of salt-stripped centrosomes, we fractionated *Drosophila* embryo extracts and assayed for centrosome complementing activity (Kawaguchi and Zheng, 2004). We found that 3% PEG precipitation could enrich for both γ TuRC and other proteins necessary for centrosome-complementing activity (see figure 4 in Kawaguchi and Zheng, 2004 for details of fractionation). Fractionation of PEG-precipitated proteins on a sucrose gradient resulted in further enrichment of the centrosome-complementing activity (see figure 4 in Kawaguchi and Zheng, 2004). Sucrose fractions capable of complementing salt-stripped centrosomes were further purified using Mono S chromatography, allowing for additional enrichment of this activity (Figure 1A; see *Materials and Methods*; also see figure 4 in Kawaguchi and Zheng, 2004). Attempts to further fractionate the complementing activity by additional chromatography schemes including anionic exchangers, hydrophobic chromatography, or dye affinity chromatography led to complete loss of activity (data not shown). Therefore, we analyzed the protein components of the active fraction obtained after the three-step purification by using MudPIT.

Mass spectrometry analyses identified 365 proteins in this active fraction (Supplemental Table S1). Consistent with the enrichment for microtubule nucleating activities, there were a large number of identified peptides corresponding to components of γ TuRC, Minispindles, and D-TACC (Supplemental Table S1). Overall, 70 (18%) of the identified proteins have known or predicted functions in directly regulating mitosis, microtubules, or the cytoskeleton (Figure 1B). Furthermore, 81 (22%) of the 365 proteins have been previously implicated in mitosis. This number includes 56 (27%) of the 205 identified components of the recent *Drosophila* whole-genome screen for mitotic regulators (Goshima *et al.*, 2007) (Figure 1C and Supplemental Table S1). The remaining 25 proteins have well-established mitotic functions based on previous studies (Supplemental Table S1). Therefore, our purification strategy had successfully enriched for mitotically relevant proteins. Other proteins identified in the centrosome-complementing fraction have no known centrosome or mitotic functions, including a large number of proteins with functions in DNA/RNA binding or regulation (Figure 1B and Supplemental Table S1). Sixty (16%) of the identified proteins were poorly characterized or uncharacterized. Because established mitotic factors were enriched in the centrosome-complementing fraction, we reasoned that further analysis of all the proteins in this fraction with an unbiased, high-resolution RNAi-based phenotypic screen could lead to identification of novel mitotic regulators that were missed in previous screens.

Identification of Novel Proteins Required for Mitosis Using an RNAi-based Phenotypic Screen

The *Drosophila* RNAi library (release 1.0; Open Biosystems, Huntsville, AL) contained cDNAs corresponding to 255 of the 365 candidate mitotic regulators we identified. Therefore, we focused our screen on these 255 proteins. The relatively short list of candidate mitotic regulators permitted us to undertake a manual RNAi screen using *Drosophila* S2 cells by analyzing defects in centrosomes, spindles, chromosomes, and cytokinesis in detail, by using strategies described by a recent RNAi screen of total *Drosophila* kinases (Bettencourt-Dias *et al.*, 2004). Briefly, defects in the four categories were further divided into 18 subcategories to facilitate analyses (Table 1 and Supplemental Figure S1A). Chromosomes, microtubules, and centrosomes were visualized using DAPI and antibodies to tubulin and CP309 (Kawaguchi and Zheng, 2004), respectively (see representative phenotypes in Supplemental Figure S1). Control RNAi experiments were conducted using dsRNA with sequence specific to EGFP (see *Materials and Methods*). Each RNAi treatment was done double-blind, and >4000 S2 cells were counted, totaling 150–250 mitotic cells. Individual mitotic cells were examined and scored for any observed phenotype. Because S2 cells have high rates of background mitotic defects, we included multiple controls in each round of RNAi treatments. Control RNAi experiments were compared with other controls from both same-day and neighboring-day experiments to generate a distribution of control phenotypic scores (PSs) that reflected the variation of background mitotic defects (see *Materials and Methods* for the definition of PSs). This distribution was used to calculate the 95 and 99% CI for defects (as reported by PS value) observed in control experiments.

We defined a mitotic regulator as a gene displaying consistent defects above the 95% CI in at least one phenotypic category. In the first round of RNAi screening, we identified 86 genes that produced mitotic defects at >95% CI in at least

Figure 1. Biochemical fractionation and proteomic analysis of centrosome-complementing activity. (A) Schematic for the biochemical enrichment of the centrosome-complementing activity. Crude extract made from *Drosophila* early embryos were first precipitated using 3% PEG, and the pellet was resuspended in buffer and fractionated on a sucrose gradient. The fractions with centrosome-complementing activity were pooled and subjected to Mono S chromatography for further purification of proteins that restore microtubule nucleation capacity to salt-stripped centrosomes. The crude extracts and certain fractions from each purification step above restored the ability of the KI-stripped *Drosophila* centrosomes to nucleate microtubules from purified tubulin (see Kawaguchi and Zheng, 2004; see Figure 4 for fraction analysis). (B) Identification of proteins present in the active fraction after Mono S chromatography by mass spectrometry. In total, 365 proteins were identified and grouped by their known or predicted functions (see Supplemental Table 1 for more details). (C) Representative proteins within the centrosome-complementing fraction that are known to associate with centrosomes or were identified as mitotic regulators by a recent whole-genome screen (Goshima *et al.*, 2007).

one phenotypic category. Twenty-four of these genes had been identified in another recent S2 cell screen (Goshima *et al.*, 2007). Therefore, we repeated the RNAi on the remaining 62 genes to rule out false positives. Through these analyses, we identified a total of 46 genes that seem to function in mitosis (Table 1). Thirty-three of these genes had been previously identified as mitotic regulators. Of the 13 novel mitotic regulators identified, five are of unknown function, two are known to function in regulating actin, and six are known regulators of transcription or translation (Table 1).

Requirement of Pontin for Mitotic Progression in Cultured Cells

One common outcome of our RNAi screen and previous large-scale RNAi screens is the identification of mitotic regulators with well-established or predicted functions in translation, chromatin remodeling, RNA processing, or transcription. Roughly 37% of the genes we identified and ~46% of the genes identified by the recent whole genome RNAi screen in S2 cells (Goshima *et al.*, 2007) fell into this category. Similarly, among the mitotic regulators identified by a RNAi screen of ~18,000 human genes, ~21% had known or predicted functions in translation, transcription, or RNA processing (Kittler *et al.*, 2004; Kittler *et al.*, 2007). A simple explanation for the high

representation of these factors is that the observed mitotic defects are indirectly caused by a lack of transcription, splicing, or translation of mitotic regulators. However, it is also possible that at least some of these genes might directly regulate mitosis in a manner independent of their functions in transcription, mRNA splicing, or translation.

We explored this possibility by performing in-depth analyses of Pontin, one of the 13 novel mitotic regulators we identified. Pontin and Reptin are highly conserved, related AAA+ ATPases that function in chromatin remodeling, DNA repair, and transcription (reviewed in Gallant, 2007). However, Pontin and Reptin also have been shown to localize to mitotic spindles and spindle poles in mammalian tissue culture cells, suggesting that these proteins might also function in mitosis (Gartner *et al.*, 2003; Sigala *et al.*, 2005). We first verified that knockdown of Pontin indeed caused mitotic defects in S2 cells. In three independent Pontin RNAi experiments performed, we observed reproducible spindle and centrosome defects (Figure 2). The spindle defects included splayed spindle poles (SSP) and abnormal spindles (AS) with either an elongated morphology and/or with dim microtubule staining. The most notable centrosome defects were the reduction of centrosome number to 1 or no centrosome in mitotic cells (CNL) or displacement of centrosomes from the spindle poles (Figure 2, CPD). Whereas RNAi-mediated down-regula-

Figure 2. Effects of Pontin reduction by RNAi in mitotic S2 cells. (A) Examples of spindle and centrosome defects caused by Pontin depletion. S2 cells treated with Pontin RNAi were fixed and stained by DAPI (blue) and antibodies against α -tubulin (red) or CP309 (marking the centrosomes green). Shown are cells exhibiting spindles with disorganized or splayed poles (SSP), elongated and/or dim bipolar spindles (in the abnormal spindle, AS, category), misplaced centrosomes (CPD) relative to spindle poles, and/or low centrosome number (CNL). (B–D) Graphs of PSs. Pontin RNAi resulted in consistent (3 independent double-blind RNAi experiments; pink arrows) spindle defects (B), low centrosome number (C), and centrosome positioning defects (D) above the 95% CI (red lines). No consistent mitotic defects were seen in Reptin RNAi-treated cells (two independent double-blind RNAi experiments; yellow arrows). Images were acquired on a Leica SP5 confocal microscope at 1024×1024 resolution with two-line averaging. Bar, 5 μ m.

tion of Pontin resulted in a strong mitotic defect, we found that RNAi of Reptin did not exhibit such defects (Figure 2, B–D).

Because the phenotypes observed by RNAi of Pontin or Reptin could be a unique phenomenon to S2 cells or could be caused by off-target effects, we further studied whether human Pontin and Reptin have mitotic functions in mammalian tissue culture cells. To facilitate analysis, we first generated affinity-purified antibodies to Pontin and Reptin (see *Materials and Methods*). Treatment of HeLa cells with three different siRNA oligos reproducibly lowered protein levels of Pontin by 65–75%, whereas two siRNA oligos diminished Reptin protein levels by 85–95% at 72 h after transfection (see examples of Western blotting analyses in Figure 3A).

To examine potential mitotic defects in live cells, we performed siRNA treatments in a HeLa cell line expressing a green fluorescent protein (GFP)-tagged Histone H2B. As Pontin and Reptin protein levels began to decline 24 h post-transfection, we performed time-lapse imaging of the cells for 72 h beginning 24 h after siRNA transfection. Reduction of Pontin resulted in an increase in mitotic cell death and a corresponding decrease in successful mitotic cell divisions (Figure 3B). We also found a large increase in the proportion of HeLa cells that died in interphase after treatment with either Pontin or Reptin oligos compared with controls (Figure 3B). Many cells treated with Pontin siRNA first arrested in mitosis for 410 ± 280 min with partially aligned chromosomes before initiating cell death, as judged by DNA fragmentation and cellular blebbing (Figure 3, C

and D). These analyses suggest that Pontin might regulate mitotic progression in HeLa cells.

Consistent with previous studies (Gartner *et al.*, 2003), we found that Pontin is localized to centrosomes at spindle poles in HeLa, U2OS, and MCF-10A cells when viewed by immunofluorescence (see examples of Pontin localization in U2OS cells in Figure 4A). Such localization was abrogated upon siRNA-mediated reduction of Pontin (Figure 4A). Because previous studies have shown that Pontin interacts with γ -tubulin in mammalian tissue culture cell lysates (Gartner *et al.*, 2003), we examined whether Pontin might play a role in tethering γ -tubulin to mitotic spindles. RNAi-mediated reduction of Pontin in U2OS cells by 65–75% resulted in an elevated cytoplasmic γ -tubulin staining and a corresponding reduction of γ -tubulin staining on spindle microtubules and at spindle poles (Figure 4, B and E). TUBGCP3, another component of the γ TuRC was also diminished at poles of bipolar spindles in Pontin-depleted cells (Supplemental Figure S2). Although staining of γ TuRC components was diminished after Pontin RNAi, recruitment of pericentrin to spindle poles did not seem to be affected. Instead, in cells treated with Pontin-specific oligos, pericentrin was less focused, often stretched along the length of spindle poles (Figure 4C). Furthermore, compared with cells treated with control or Reptin oligos, reduction of Pontin using three different oligos resulted in an increase of spindles with multiple spindle poles, or reduced/disorganized microtubule morphology (Figure 4, A–C, and quantified in D). Further displacement of γ TuRC components was evident on spindles with aberrant morphology, whereas pericentrin was usually localized to most spin-

Figure 3. Effects of reduction of Pontin and Reptin on mammalian cell division and survival. (A) Reduction of Pontin and Reptin in HeLa cells by siRNA. Lysates made from HeLa cells treated for 72 h with control siRNA or siRNAs targeting Pontin and Reptin were fractionated by SDS-polyacrylamide gel electrophoresis in decreasing amounts (16, 12, or 8 μ l) and immunoblotted for the indicated proteins. RNAi treatment resulted in ~65–75% or 85–95% reduction of Pontin or Reptin, respectively, compared with controls. (B–D) Analyses of Pontin and Reptin RNAi by live imaging. siRNA-treated HeLa cells expressing Histone H2B-GFP were imaged for 72 h beginning 24 h after transfection. Graph in B shows percentages of cells completing division, dying in mitosis, or dying in interphase after Pontin, Reptin, or control siRNA treatment. Error bars, standard deviations calculated from at least four independent experiments where at least 30 mitotic events were analyzed in each experiment. Whereas both Pontin and Reptin siRNA-treatment caused interphase cell death, only Pontin siRNA caused mitotic cell death. Graph in C shows that Pontin RNAi-treated cells that died in mitosis first underwent mitotic arrest and then committed death, whereas the cells that divided successfully had similar mitotic timing as control cells. Error bars, standard deviations calculated from three independent experiments where at least 50 mitotic events were analyzed in each experiment. The mitotic timing was calculated from nuclear envelope breakdown to the onset of anaphase or cell death. (D) Still images of control or Pontin siRNA-treated HeLa cells entering mitosis. Brightfield and fluorescence images captured at indicated time frame (hours:minutes) are shown. Time set to “0:00” upon nuclear envelope breakdown. Note that Pontin-depleted cells arrest in prometaphase with partially aligned chromosomes (arrows) before undergoing mitotic death, judged by cell membrane blebbing and DNA compaction (arrowheads). Bar, 10 μ m.

dle poles (Figure 4, B and C). Because Western blotting showed that the total cellular level of γ -tubulin remained the same in cells treated with either Pontin oligos or control oligos (Supplemental Figure 3), our findings suggest that human Pontin might regulate the localization of γ TuRC to the mitotic spindle.

In contrast, upon RNAi induced reduction of Reptin, we observed neither mitotic defects nor displacement of γ -tubulin from the spindle microtubules (Figure 4, D and E). Our Reptin antibodies also failed to stain spindles or spindle poles (data not shown). However, in cells depleted of both Pontin and Reptin by RNAi, we observed enhanced spindle defects in

fixed cells (Figure 4D) and increased mitotic death in live cell imaging (Supplemental Figure S3). Similar spindle defects were observed following Pontin and/or Reptin RNAi in U2OS, HeLa, and MCF-10A human cell lines. Therefore, consistent with our RNAi analyses of S2 cells, Pontin seemed to regulate mitotic spindle assembly in human cells, whereas Reptin depletion alone did not result in mitotic defects. The enhancement of mitotic defects observed in human cells after knockdown of both Pontin and Reptin suggests that Reptin may play an auxiliary function to regulate/enhance Pontin activity.

Figure 4. Effects of Pontin reduction on mitotic spindle assembly in mammalian tissue culture cells. (A) Spindle pole localization of Pontin. U2OS cells treated with either control or Pontin siRNA were fixed in methanol and stained by DAPI (blue), antibodies to α -tubulin (red), and peptide antibodies to Pontin (green). Note that the spindle pole staining of Pontin (top) diminished after treatment with Pontin siRNA (bottom). Images were acquired on a Nikon E800. (B) Displacement of γ -tubulin from mitotic spindles after Pontin depletion. γ -Tubulin staining (green) in Pontin-depleted cells was reduced both at the mitotic spindle poles and along the spindles with a corresponding increase in cytoplasmic staining (bottom), relative to control-treated cells (top). Images shown are maximum projections of z-stacks acquired on a Yokogawa CSU10 spinning disk confocal microscope. (C) Spindle defects in Pontin-depleted cells. Spindle pole fragmentation and multipolar spindles were the most frequent defects observed after Pontin depletion, as viewed by pericentrin staining (green). Images were acquired on a Nikon E800. (D) Quantification of abnormal and multipolar spindles in U2OS cells 48–72 h after control, Pontin (3 oligos), or Reptin (2 oligos) siRNA treatment. Similar increases in spindle defects after Pontin reduction were observed in HeLa and MCF10-A cells (data not shown). (E) Quantification of the ratio of γ -tubulin intensity at bipolar spindle poles to the intensity of a representative area of the cytoplasm (see *Materials and Methods*). Error bars in D and E represent SD from at least three independent experiments where >50 mitotic cells or >30 mitotic poles were counted or measured in each experiment, respectively. Bars, 10 μ m.

Pontin Directly Regulates Microtubule Assembly and Organization in Mitosis

Pontin and Reptin are known to regulate a variety of cellular processes in interphase including transcription, chromatin remodeling, and DNA repair. Although the two proteins are known to function cooperatively in the same complex in many settings, they have also been shown to function either antagonistically or independently of each other in interphase (Bauer *et al.*, 2000; Cho *et al.*, 2001; Bellosta *et al.*, 2005). Due to the complicated interphase roles of Pontin and Reptin, it is difficult to assess whether they have direct roles in regulating mitosis solely by using RNAi-based studies in tissue culture cells.

To determine whether Pontin has a mitosis-specific function in regulating microtubule assembly and organization,

we turned to *Xenopus* egg extracts made from cytotstatic factor-arrested mature oocytes (Murray, 1991). *Xenopus* egg extract supports spindle assembly in the presence of sperm chromatin (Sawin and Mitchison, 1991), RanGTP (Carazo-Salas *et al.*, 1999; Kalab *et al.*, 1999; Ohba *et al.*, 1999; Wilde and Zheng, 1999; Zhang *et al.*, 1999), or RanGTP plus magnetic beads coated with the mitotic kinase Aurora A (AurA) (Tsai and Zheng, 2005). Because spindle assembly in meiotically arrested *Xenopus* egg extracts occurs independently of the prior interphase, mitosis-specific roles of any protein can be examined separately from interphase activities.

Using antibodies that specifically recognize *Xenopus* Pontin on Western blots of egg extracts (Figure 5A), we found that Pontin was not only concentrated at spindle poles but also was faintly localized along the microtubules of spindles

Figure 5. Interactions among Pontin, Reptin, and γ TuRC in *Xenopus* egg extracts. (A) Specificity of antibodies generated against a peptide of *Xenopus* Pontin (Pep-Pontin) and full-length Pontin (FL-Pontin) and Reptin (FL-Reptin) by Western blotting analysis using *Xenopus* egg extracts. (B) Localization of Pontin on spindles. RanGTP-induced spindles in *Xenopus* egg extracts were fixed in methanol and stained with anti- α -tubulin (red) and peptide anti-Pontin (green) antibodies. Pontin localized to spindle poles and lightly along spindle microtubules. (C) Interaction between Pontin and γ TuRC in egg extracts. Control IgG, Pontin, or γ -tubulin antibodies were used in immunoprecipitation reactions. Pontin and γ -tubulin immunoprecipitated each other reciprocally. Each antibody also coprecipitated Xgrip109 and Xgrip210, other components of γ TuRC. (D) Comigration of Pontin and Reptin in *Xenopus* egg extracts on sucrose gradients. Protein standards with S values of 4.3 S (bovine serum albumin), 7.3 S (rabbit muscle aldolase), 11.3 S (bovine liver catalase), and 19.4 S (porcine thyroglobulin) were run on identical gradients. Peaks corresponding to these protein standards are indicated (arrowheads). (E) Copurification of Pontin and Reptin with γ TuRC. Purified γ TuRC complex (Coomassie Blue stained; see Materials and Methods) contained substoichiometric amounts of both Pontin and Reptin as revealed by Western blotting analysis. (F) Reptin antibodies fail to immunoprecipitate the Reptin associated with γ TuRC in *Xenopus* egg extracts. *Xenopus* egg extracts were either mock-depleted using unimmunized rabbit IgG or depleted of Reptin (and associated Pontin) using Reptin-specific antibodies. Different amounts of egg extracts (0.75, 0.25, and 0.1 μ l) were analyzed by Western blotting (top). Approximately 85–95% of the total pool of Reptin (and Pontin; data not shown) was depleted. After IgG or Reptin depletion, γ TuRC was immunoprecipitated from egg extracts using γ -tubulin antibodies. The immunoprecipitates were analyzed by loading decreasing amounts of sample (12, 8, and 4 μ l) on the gel. The same amount of Pontin and Reptin remained associated with the γ TuRC in Reptin depleted egg extracts as compared with mock-depleted ones (bottom), demonstrating that Reptin antibodies failed to recognize the γ TuRC-associated Reptin in the egg extracts. Bar, 10 μ m.

(see examples of Pontin localization on spindle poles and microtubules stimulated by RanGTP in Figure 5B). Consistent with our observation in tissue culture cells, anti-Reptin antibodies (Figure 5A) did not show spindle localization (data not shown). Because GST-tagged Pontin protein can pull-down γ -tubulin in human U937 cell lysates (Gartner *et al.*, 2003), we asked whether Pontin interacted with γ TuRC in *Xenopus* egg extracts. Reciprocal immunoprecipitation using antibodies to Pontin or γ -tubulin showed that Pontin interacted with γ TuRC in egg extracts (Figure 5C). Because we found that Pontin and Reptin cofractionated on sucrose gradients (Figure 5D) and coimmunoprecipitated each other (Figure 6A), we asked whether Reptin was also present in γ TuRC. Western blotting showed that purified γ TuRC contained both Pontin and Reptin (Figure 5E). This prompted us to examine why Reptin was not localized on spindles similarly to Pontin. We reasoned that our Reptin antibody might not recognize the small pool of Reptin present in the γ TuRC. This was likely because quantitative Western analysis estimated the levels of Pontin and Reptin in egg extracts at ~ 1 μ M each, a concentration that is 100- to 1000-fold more abundant than the estimated γ TuRC concentration (Stearns and Kirschner, 1994; Zheng *et al.*, 1995). Using the Reptin antibody, we removed the majority of Reptin (90–95%) from the egg extracts and then immunoprecipitated γ TuRC from

this egg extract. We found that a similar amount of Pontin and Reptin was associated with γ TuRC after depletion (Figure 5F). Therefore, we concluded that our Reptin antibody seemed not to recognize the pool of Reptin that associates with γ TuRC by immunofluorescence or immunoprecipitation. Additionally, the stable pool of Reptin/Pontin interacting with γ TuRC after removal of excess Reptin/Pontin suggested a specific interaction of these proteins that cannot be explained by contamination of purified γ TuRC (Figure 5E).

Because only the Pontin antibody could deplete the pool of Pontin and Reptin associated with γ TuRC, we used this antibody to deplete Pontin and Reptin from *Xenopus* egg extracts (Figure 6A). IgG from unimmunized rabbits was used for mock depletion. We found that immunodepleting Pontin resulted in a 90–95% depletion of both Pontin and Reptin as well as 70–80% codepletion of γ TuRC components (Figure 6A). Spindle assembly was induced using AurA-beads and RanGTP in these depleted egg extracts. Because AurA-beads function as microtubule organization centers in egg extracts in the presence of RanGTP (Tsai and Zheng, 2005), we could assess the ability of the egg extracts to support microtubule assembly and organization by examining the brightness and organization of microtubules assembled around the beads. Compared with mock-depleted egg extracts, extracts depleted using Pontin antibodies could not

Figure 6. Requirement for Pontin in microtubule assembly in *Xenopus* egg extracts. (A) Western blotting analyses of egg extracts that were mock depleted (–IgG), depleted with Pontin antibodies (–Pontin), depleted of Pontin followed by γ TuRC addition (–Pontin+ γ TuRC), or depleted of Pontin followed by addition of γ TuRC, Pontin, and Reptin (–Pontin+ γ TuRC+Pont/Rept). Immunodepletion of Pontin from the egg extracts codepleted both γ TuRC components and Reptin. Endogenous levels of γ TuRC, Pontin, and Reptin were restored by addition of purified proteins. Effects of protein depletion on microtubule assembly are assayed in C. (B) Examples of AurA beads associated with a spindle-like structure (arrow), bright microtubule asters (filled arrowheads), faint microtubule asters (open arrowheads), or no microtubules. (C) Quantification of AurA-beads associated with spindles or bright microtubule asters in egg extracts treated as in A. Microtubule structures in controls were normalized to 100%.

Depletion of Pontin abrogated the capacity of AurA beads to stimulate assembly of spindles and microtubule asters as compared with mock depletion. Addition of purified γ TuRC resulted in partial rescue. Full rescue of microtubule nucleation was only observed upon addition of purified Pontin and Reptin along with γ TuRC. Error bars, SD from >10 independent experiments. The p values were calculated using a two-tailed Student's t test. Bar, 10 μ m.

support the assembly of bright astral or spindle microtubule arrays around the AurA-beads (Figure 6, B and C). These defects could only be partially rescued by adding back purified γ TuRC (Figure 6, A and C).

Next, we determined whether add-back of purified Pontin could further rescue microtubule assembly. Consistent with previous findings, the bacterially expressed Pontin predominantly existed as a monomeric protein (Puri *et al.*, 2007). We found that addition of this monomeric Pontin together with purified γ TuRC resulted in a similar partial rescue of microtubule assembly as the addition of γ TuRC alone (data not shown). We reasoned that the lack of rescue might be due to the lack of activity of the bacterially expressed Pontin. Indeed, coexpression of both Pontin and Reptin in bacteria was shown previously to stimulate the ATPase activities of both proteins and induce the formation of stacked, double hexameric ring complexes of Pontin and Reptin (Ikura *et al.*, 2000; Puri *et al.*, 2007). To make the purified Pontin/Reptin complex, we took two approaches. In the first approach, we expressed *Xenopus* His₆-tagged Pontin and His₆-tagged Reptin individually, and then we mixed the bacterial lysates together and copurified the two proteins. Alternatively, human His₆-tagged Pontin and Reptin were expressed together using a bicistronic expression construct (Puri *et al.*, 2007) and purified. We found that addition of the Pontin and Reptin complex produced by either of the two methods together with γ TuRC resulted in a significantly better rescue than adding γ TuRC alone (Figure 6, A and C). These analyses suggested that the complex of Pontin and Reptin has mitosis-specific functions in regulating microtubule assembly and organization.

DISCUSSION

Proteome and RNAi-based Identification of Candidate Mitotic Regulators

Using an activity-based assay and biochemical fractionation, we have enriched for proteins from *Drosophila* early embryos that could restore microtubule nucleation of salt-stripped

Drosophila centrosomes. The enrichment process resulted in identification of a short list of candidate mitotic regulators, which allowed us to carry out in-depth RNAi-based phenotypic analyses to identify mitotic regulators. Our screen differs from the recent whole-genome screen in S2 cells (Goshima *et al.*, 2007) in several aspects. First, to capture sufficient numbers of mitotic cells using an automated imaging system, the whole genome screen used RNAi of Cdc27 to silence the anaphase-promoting complex (APC) and enrich for metaphase cells, whereas we did not inhibit APC in our screen. In addition, whereas we used a 100 $\times$ oil lens in our manual inspection of mitotic cells, a 40 $\times$ dry lens was used to capture images in the whole genome analysis. Finally, we manually inspected more categories of mitotic defects than that of the whole-genome screen. Consequently, our screen could offer higher resolution.

From a total of 255 genes analyzed, we identified 46 regulators of mitosis, 13 of which were novel. It is interesting to note that both Pontin and Reptin were identified in the whole-genome screen as having a weak mitotic phenotype after RNAi treatment, and they were therefore not included in the final list of mitotic regulators (Goshima *et al.*, 2007). However, in our screen, we found that Pontin RNAi caused strong mitotic defects in S2 cells. We were able to confirm this by carrying out RNAi analyses in three different mammalian cell lines. Therefore, our studies demonstrate the utility of coupling functional assays with biochemical fractionation and a low-throughput RNAi screen to identify new mitotic regulators that could be missed by large-scale analyses.

The Mitosis-Specific Function of Pontin and Reptin in Regulating Microtubule Assembly via γ TuRC

In this study, we identified both Pontin and Reptin, two proteins with well-established functions in chromatin remodeling, to be present in the centrosome-complementing fraction. Using *Xenopus* egg extract, we showed that both Pontin and Reptin interact with γ TuRC and that they are required for the egg extract to nucleate and organize robust

microtubule arrays. This suggests that both proteins have a mitosis-specific function in regulating the microtubule cytoskeleton. Consistent with this, reduction of Pontin in tissue culture cells resulted in increased spindle defects. However, we believe it is likely that not all of the mitotic defects observed in Pontin RNAi-treated cells were solely due to deregulation of microtubules in mitosis. For example, the mitotic cell death phenotype we observed cannot be simply explained by spindle defects alone, because defective spindle assembly often triggers the spindle checkpoint, typically leading to a more prolonged mitotic arrest in the cell lines we used (reviewed in Rieder and Maiato, 2004). Mitotic death (also referred to as mitotic catastrophe) can result from imbalanced transcription of apoptotic regulators or from irresolvable DNA damage (Rieder and Maiato, 2004; Blagosklonny, 2007). We have recently shown that reduction of RanBP1, a regulator of the Ran system, also caused mitotic cell death, likely due to combined defects in mitotic spindle assembly and spindle checkpoint signaling (Li *et al.*, 2007). Because Pontin is a component of many interphase complexes, including those involved in chromatin function, we suggest that the mitotic cell death phenotype could be caused by a combination of cellular defects, including defects in spindle formation and chromatin organization due to lack of Pontin. In this context, it will be interesting to analyze whether Pontin reduction results in DNA damage or disorganization of centromeric DNA (which could affect kinetochore functions), and whether this contributes to the mitotic cell death we observe.

Interestingly, whereas RNAi-mediated depletion of Pontin in *Drosophila* and in different human cell lines caused defective spindle assembly and mitotic cell death, similar reduction of Reptin did not affect mitosis. However, Reptin depletion was able to enhance the mitotic defects observed after Pontin knockdown. One possible explanation is that Reptin functions together with Pontin to regulate microtubule assembly, but the different functions that Pontin and Reptin perform in interphase could result in different mitotic phenotypes. Consistent with this, although Pontin and Reptin are related ATPases and they are found together in several chromatin remodeling complexes, they do not always function in the same manner in interphase. In fact, expression levels of these proteins are not always similar, and certain protein complexes contain only one of these proteins (Etard *et al.*, 2000; Cho *et al.*, 2001; Ghaemmaghami *et al.*, 2003). Similarly, it has been shown that overexpression of Pontin in tissue culture cells can displace Reptin from the transcription factor c-Myc (Bellosta *et al.*, 2005). Furthermore, Pontin and Reptin oppose one another to control transcription in the β -catenin–TCF pathway in both *Drosophila* and zebrafish (Bauer *et al.*, 2000; Rottbauer *et al.*, 2002). Therefore, knockdown of Reptin alone might affect Pontin-independent pathways in interphase that obscure any mitotic phenotype or preclude entry into mitosis. Alternatively, whereas both Pontin and Reptin are needed for the full rescue of microtubule assembly in *Xenopus* egg extracts, it is possible that the mitotic function of Reptin in assisting Pontin assembly is masked by other proteins in tissue culture cells. Currently, we cannot distinguish between these two possibilities.

How might Pontin and Reptin regulate microtubule assembly? Both Pontin and Reptin are members of the AAA+ family of ATPases, which are typically involved in regulating protein–protein and protein–DNA interactions in many cellular contexts (Neuwald *et al.*, 1999; Iyer *et al.*, 2004; Hanson and Whiteheart, 2005). Consistent with a general role in assembly of diverse cellular complexes, Pontin and Reptin

have been shown to transiently associate with U3 box C/D pre-small nucleolar ribonucleoproteins (Newman *et al.*, 2000; King *et al.*, 2001; Watkins *et al.*, 2002; McKeegan *et al.*, 2007) and the Ino80 chromatin remodeling complex (Jonsson *et al.*, 2004) in a manner that is essential for the full assembly of each complex. Interestingly, Pontin and Reptin are also rapidly up-regulated in response to flagellar reassembly in *Chlamydomonas*, a process that strongly increases expression of many microtubule-related factors, heat-shock proteins, and tubulin chaperones (Stolc *et al.*, 2005). In this context, it is tempting to speculate that Pontin, and possibly Reptin, may function as chaperones to facilitate microtubule assembly by transiently interacting with γ TuRC. Such a chaperone function could facilitate the localization of γ TuRC to both spindle poles and along spindle microtubules.

Are There Additional Proteins with Known Functions in Interphase That Also Directly Regulate Mitosis?

In addition to known mitotic proteins, our RNAi screen also identified proteins with established interphase roles in regulating proper functions of DNA or RNA. This group includes proteins that regulate transcription, chromatin remodeling, DNA repair, RNA processing, or protein translation (Table 1). For the convenience of discussion, we refer to all proteins belonging to this group as DNA/RNA related. Interestingly, of the 205 mitotic regulators identified by the whole genome screen, ~46% belong to this group (Goshima *et al.*, 2007). Similarly, ~21% of the candidate mitotic regulators identified by screening ~18,000 human genes have known functions in DNA/RNA-related processes (Kittler *et al.*, 2004). One possible interpretation for the identification of these factors by RNAi analyses is to assume that cell division defects observed after depletion of these factors are an indirect consequence of defects in interphase gene expression or protein translation.

However, findings presented here along with several previous studies suggest that at least some of these proteins might have mitosis-specific roles that are independent of their functions in interphase. For example, it has been demonstrated that spindle assembly requires mRNA export factor Rae1, and bulk RNA (Blower *et al.*, 2005). This suggests that some species of RNA and their associated factors may play direct roles in regulating mitosis. Likewise, BRCA1 and BARD1, which have well-established roles in DNA repair, also have centrosome and mitosis-specific functions (reviewed in Parvin and Sankaran, 2006), suggesting that some of the DNA repair proteins identified by various RNAi screens of mitotic regulators might also play a role in regulating spindle assembly. In light of these studies, we believe that it is important to consider that DNA/RNA-related factors identified as candidate mitotic regulators may possess genuine mitosis-specific functions. Increased knowledge of such factors may help us to better understand both mitotic regulation and how mitosis is integrated with interphase cellular processes.

While this manuscript was in review, a functional interaction between Pontin (RuvBL1) and integrin-linked kinase (ILK) was reported (Fielding *et al.*, 2008). Here, the authors demonstrate that ILK and Pontin exist in a complex that is important for spindle organization and that also contains α -tubulin, β -tubulin, and ch-TOG. ILK localization to mitotic spindle poles is dependent on Pontin, indicating that Pontin may be involved in recruiting multiple complexes to the centrosome. It is therefore possible that the mitotic death phenotype we observed following Pontin depletion may be a consequence of deregulation of multiple complexes required for centrosome integrity and mitotic progression.

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REFERENCES

- Bauer, A., Chauvet, S., Huber, O., Usseglio, F., Rothbacher, U., Aragnol, D., Kemler, R., and Pradel, J. (2000). Pontin52 and reptin52 function as antagonistic regulators of beta-catenin signalling activity. *EMBO J.* 19, 6121–6130.
- Bellosta, P., Hulf, T., Balla Diop, S., Usseglio, F., Pradel, J., Aragnol, D., and Gallant, P. (2005). Myc interacts genetically with Tip48/Reptin and Tip49/Pontin to control growth and proliferation during *Drosophila* development. *Proc. Natl. Acad. Sci. USA* 102, 11799–11804.
- Bettencourt-Dias, M., et al. (2004). Genome-wide survey of protein kinases required for cell cycle progression. *Nature* 432, 980–987.
- Blagosklonny, M. V. (2007). Mitotic arrest and cell fate: why and how mitotic inhibition of transcription drives mutually exclusive events. *Cell Cycle* 6, 70–74.
- Blower, M. D., Nachury, M., Heald, R., and Weis, K. (2005). A Rae1-containing ribonucleoprotein complex is required for mitotic spindle assembly. *Cell* 121, 223–234.
- Cao, K., Nakajima, R., Meyer, H. H., and Zheng, Y. (2003). The AAA-ATPase Cdc48/p97 regulates spindle disassembly at the end of mitosis. *Cell* 115, 355–367.
- Carazo-Salas, R. E., Guarguaglini, G., Gruss, O. J., Segref, A., Karsenti, E., and Mattaj, I. W. (1999). Generation of GTP-bound Ran by RCC1 is required for chromatin-induced mitotic spindle formation. *Nature* 400, 178–181.
- Cho, S. G., Bhoomik, A., Broday, L., Ivanov, V., Rosenstein, B., and Ronai, Z. (2001). TIP49b, a regulator of activating transcription factor 2 response to stress and DNA damage. *Mol. Cell Biol.* 21, 8398–8413.
- Echalier, G. (1997). *Drosophila* Cells in Culture, San Diego, CA: Academic Press.
- Ems-McClung, S. C., Zheng, Y., and Walczak, C. E. (2004). Importin α/β and Ran-GTP regulate XCTK2 microtubule binding through a bipartite nuclear localization signal. *Mol. Biol. Cell* 15, 46–57.
- Etard, C., Wedlich, D., Bauer, A., Huber, O., and Kuhl, M. (2000). Expression of *Xenopus* homologs of the beta-catenin binding protein pontin52. *Mech. Dev.* 94, 219–222.
- Fielding, A. B., Dobreva, I., McDonald, P. C., Foster, L. J., and Dedhar, S. (2008). Integrin-linked kinase localizes to the centrosome and regulates mitotic spindle organization. *J. Cell Biol.* 180, 681–689.
- Foley, E., and O'Farrell, P. H. (2004). Functional dissection of an innate immune response by a genome-wide RNAi screen. *PLoS Biol.* 2, E203.
- Gallant, P. (2007). Control of transcription by Pontin and Reptin. *Trends Cell Biol.* 17, 187–192.
- Gartner, W., Rossbacher, J., Zierhut, B., Daneva, T., Base, W., Weissel, M., Waldhausl, W., Pasternack, M. S., and Wagner, L. (2003). The ATP-dependent helicase RUVBL1/TIP49a associates with tubulin during mitosis. *Cell Motil. Cytoskeleton* 56, 79–93.
- Ghaemmaghami, S., Huh, W. K., Bower, K., Howson, R. W., Belle, A., Dephoure, N., O'Shea, E. K., and Weissman, J. S. (2003). Global analysis of protein expression in yeast. *Nature* 425, 737–741.
- Goshima, G., Wollman, R., Goodwin, S. S., Zhang, N., Scholey, J. M., Vale, R. D., and Stuurman, N. (2007). Genes required for mitotic spindle assembly in *Drosophila* S2 cells. *Science* 316, 417–421.
- Gruss, O. J., Carazo-Salas, R. E., Schatz, C. A., Guarguaglini, G., Kast, J., Wilm, M., Le Bot, N., Vernos, I., Karsenti, E., and Mattaj, I. W. (2001). Ran induces spindle assembly by reversing the inhibitory effect of importin alpha on TPX2 activity. *Cell* 104, 83–93.
- Gunawardane, R. N., Martin, O. C., Cao, K., Zhang, L., Dej, K., Iwamatsu, A., and Zheng, Y. (2000). Characterization and reconstitution of *Drosophila* gamma-tubulin ring complex subunits. *J. Cell Biol.* 151, 1513–1524.
- Gunawardane, R. N., Zheng, Y., Oegema, K., and Wiese, C. (2001). Purification and reconstitution of *Drosophila* gamma-tubulin complexes. *Methods Cell Biol.* 67, 1–25.
- Hanson, P. I., and Whiteheart, S. W. (2005). AAA+ proteins: have engine, will work. *Nat. Rev. Mol. Cell Biol.* 6, 519–529.
- Huang, P., Senga, T., and Hamaguchi, M. (2007). A novel role of phospho-beta-catenin in microtubule regrowth at centrosome. *Oncogene* 26, 4357–4371.
- Ikura, T., Ogryzko, V. V., Grigoriev, M., Groisman, R., Wang, J., Horikoshi, M., Scully, R., Qin, J., and Nakatani, Y. (2000). Involvement of the TIP60 histone acetylase complex in DNA repair and apoptosis. *Cell* 102, 463–473.
- Iyer, L. M., Leippe, D. D., Koonin, E. V., and Aravind, L. (2004). Evolutionary history and higher order classification of AAA+ ATPases. *J. Struct. Biol.* 146, 11–31.
- Jonsson, Z. O., Jha, S., Wohlschlegel, J. A., and Dutta, A. (2004). Rvb1p/Rvb2p recruit Arp5p and assemble a functional Ino80 chromatin remodeling complex. *Mol. Cell* 16, 465–477.
- Kalab, P., Pralle, A., Isacoff, E. Y., Heald, R., and Weis, K. (2006). Analysis of a RanGTP-regulated gradient in mitotic somatic cells. *Nature* 440, 697–701.
- Kalab, P., Pu, R. T., and Dasso, M. (1999). The ran GTPase regulates mitotic spindle assembly. *Curr. Biol.* 9, 481–484.
- Kalab, P., Weis, K., and Heald, R. (2002). Visualization of a Ran-GTP gradient in interphase and mitotic *Xenopus* egg extracts. *Science* 295, 2452–2456.
- Kaplan, D. D., Meigs, T. E., Kelly, P., and Casey, P. J. (2004). Identification of a role for beta-catenin in the establishment of a bipolar mitotic spindle. *J. Biol. Chem.* 279, 10829–10832.
- Kawaguchi, S., and Zheng, Y. (2004). Characterization of a *Drosophila* centrosome protein CP309 that shares homology with Kendrin and CG-NAP. *Mol. Biol. Cell* 15, 37–45.
- King, T. H., Decatur, W. A., Bertrand, E., Maxwell, E. S., and Fournier, M. J. (2001). A well-connected and conserved nucleoplasmic helicase is required for production of box C/D and H/ACA snoRNAs and localization of snoRNP proteins. *Mol. Cell Biol.* 21, 7731–7746.
- Kittler, R. et al. (2007). Genome-scale RNAi profiling of cell division in human tissue culture cells. *Nat. Cell Biol.* 9, 1401–1412.
- Kittler, R. et al. (2004). An endoribonuclease-prepared siRNA screen in human cells identifies genes essential for cell division. *Nature* 432, 1036–1040.
- Li, H. Y., Ng, W. P., Wong, C. H., Iglesias, P. A., and Zheng, Y. (2007). Coordination of chromosome alignment and mitotic progression by the chromosome-based Ran signal. *Cell Cycle* 6, 1886–1895.
- Li, H. Y., Wirtz, D., and Zheng, Y. (2003). A mechanism of coupling RCC1 mobility to RanGTP production on the chromatin in vivo. *J. Cell Biol.* 160, 635–644.
- Li, H. Y., and Zheng, Y. (2004). Phosphorylation of RCC1 in mitosis is essential for producing a high RanGTP concentration on chromosomes and for spindle assembly in mammalian cells. *Genes Dev.* 18, 512–527.
- Liska, A. J., Popov, A. V., Sunyaev, S., Coughlin, P., Habermann, B., Shevchenko, A., Bork, P., and Karsenti, E. (2004). Homology-based functional proteomics by mass spectrometry: application to the *Xenopus* microtubule-associated proteome. *Proteomics* 4, 2707–2721.
- Martin, O. C., Gunawardane, R. N., Iwamatsu, A., and Zheng, Y. (1998). Xgrip 109, a gamma tubulin-associated protein with an essential role in gamma tubulin ring complex (gammaTuRC) assembly and centrosome function. *J. Cell Biol.* 141, 675–687.
- McKeegan, K. S., Debieux, C. M., Boulon, S., Bertrand, E., and Watkins, N. J. (2007). A dynamic scaffold of pre-snoRNP factors facilitates human box C/D snoRNP assembly. *Mol. Cell Biol.* 27, 6782–6793.
- Moritz, M., Zheng, Y., Alberts, B. M., and Oegema, K. (1998). Recruitment of the gamma-tubulin ring complex to *Drosophila* salt-stripped centrosome scaffolds. *J. Cell Biol.* 142, 775–786.
- Murray, A. W. (1991). Cell cycle extracts. *Methods Cell Biol.* 36, 581–605.
- Nachury, M. V., Maresca, T. J., Salmon, W. C., Waterman-Storer, C. M., Heald, R., and Weis, K. (2001). Importin beta is a mitotic target of the small GTPase Ran in spindle assembly. *Cell* 104, 95–106.
- Neuwald, A. F., Aravind, L., Spouge, J. L., and Koonin, E. V. (1999). AAA+: a class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. *Genome Res* 9, 27–43.
- Newman, D. R., Kuhn, J. F., Shanab, G. M., and Maxwell, E. S. (2000). Box C/D snoRNA-associated proteins: two pairs of evolutionarily ancient proteins and possible links to replication and transcription. *RNA* 6, 861–879.

- O'Connell, C. B., and Khodjakov, A. L. (2007). Cooperative mechanisms of mitotic spindle formation. *J. Cell Sci.* 120, 1717–1722.
- Oegema, K., Wiese, C., Martin, O. C., Milligan, R. A., Iwamatsu, A., Mitchison, T. J., and Zheng, Y. (1999). Characterization of two related *Drosophila* gamma-tubulin complexes that differ in their ability to nucleate microtubules. *J. Cell Biol.* 144, 721–733.
- Ohba, T., Nakamura, M., Nishitani, H., and Nishimoto, T. (1999). Self-organization of microtubule asters induced in *Xenopus* egg extracts by GTP-bound Ran. *Science* 284, 1356–1358.
- Parvin, J. D., and Sankaran, S. (2006). The BRCA1 E3 ubiquitin ligase controls centrosome dynamics. *Cell Cycle* 5, 1946–1950.
- Puri, T., Wendler, P., Sigala, B., Saibil, H., and Tsaneva, I. R. (2007). Dodecameric structure and ATPase activity of the human TIP48/TIP49 complex. *J. Mol. Biol.* 366, 179–192.
- Rieder, C. L., and Maiato, H. (2004). Stuck in division or passing through: what happens when cells cannot satisfy the spindle assembly checkpoint. *Dev. Cell* 7, 637–651.
- Rottbauer, W., Saurin, A. J., Lickert, H., Shen, X., Burns, C. G., Wo, Z. G., Kemler, R., Kingston, R., Wu, C., and Fishman, M. (2002). Reptin and pontin antagonistically regulate heart growth in zebrafish embryos. *Cell* 111, 661–672.
- Royle, S. J., Bright, N. A., and Lagnado, L. (2005). Clathrin is required for the function of the mitotic spindle. *Nature* 434, 1152–1157.
- Sauer, G., Korner, R., Hanisch, A., Ries, A., Nigg, E. A., and Sillje, H. H. (2005). Proteome analysis of the human mitotic spindle. *Mol. Cell Proteomics* 4, 35–43.
- Sawin, K. E., and Mitchison, T. J. (1991). Mitotic spindle assembly by two different pathways in vitro. *J. Cell Biol.* 112, 925–940.
- Schnackenberg, B. J., Hull, D. R., Balczon, R. D., and Palazzo, R. E. (2000). Reconstitution of microtubule nucleation potential in centrosomes isolated from *Spisula solidissima* oocytes. *J. Cell Sci.* 113, 943–953.
- Schnackenberg, B. J., Khodjakov, A., Rieder, C. L., and Palazzo, R. E. (1998). The disassembly and reassembly of functional centrosomes in vitro. *Proc. Natl. Acad. Sci. USA* 95, 9295–9300.
- Sigala, B., Edwards, M., Puri, T., and Tsaneva, I. R. (2005). Relocalization of human chromatin remodeling cofactor TIP48 in mitosis. *Exp. Cell Res.* 310, 357–369.
- Sillibourne, J. E., Delaval, B., Redick, S., Sinha, M., and Doxsey, S. J. (2007). Chromatin remodeling proteins interact with pericentrin to regulate centrosome integrity. *Mol. Biol. Cell* 18, 3667–3680.
- Skop, A. R., Liu, H., Yates, J., 3rd, Meyer, B. J., and Heald, R. (2004). Dissection of the mammalian midbody proteome reveals conserved cytokinesis mechanisms. *Science* 305, 61–66.
- Sohail, M. (2005). Gene Silencing by RNA Interference: Technology and Application, Boca Raton, FL: CRC Press.
- Stearns, T., and Kirschner, M. (1994). In vitro reconstitution of centrosome assembly and function: the central role of gamma-tubulin. *Cell* 76, 623–637.
- Stolc, V., Samanta, M. P., Tongprasit, W., and Marshall, W. F. (2005). Genome-wide transcriptional analysis of flagellar regeneration in *Chlamydomonas reinhardtii* identifies orthologs of ciliary disease genes. *Proc. Natl. Acad. Sci. USA* 102, 3703–3707.
- Tabb, D. L., McDonald, W. H., and Yates, J. R., 3rd. (2002). DTASelect and Contrast: tools for assembling and comparing protein identifications from shotgun proteomics. *J. Proteome Res.* 1, 21–26.
- Tsai, M. Y., Wang, S., Heidinger, J. M., Shumaker, D. K., Adam, S. A., Goldman, R. D., and Zheng, Y. (2006). A mitotic lamin B matrix induced by RanGTP required for spindle assembly. *Science* 311, 1887–1893.
- Tsai, M. Y., and Zheng, Y. (2005). Aurora A kinase-coated beads function as microtubule-organizing centers and enhance RanGTP-induced spindle assembly. *Curr. Biol.* 15, 2156–2163.
- Vong, Q. P., Cao, K., Li, H. Y., Iglesias, P. A., and Zheng, Y. (2005). Chromosome alignment and segregation regulated by ubiquitination of survivin. *Science* 310, 1499–1504.
- Washburn, M. P., Wolters, D., and Yates, J. R., 3rd. (2001). Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nat. Biotechnol.* 19, 242–247.
- Watkins, N. J., Dickmanns, A., and Luhrmann, R. (2002). Conserved stem II of the box C/D motif is essential for nucleolar localization and is required, along with the 15.5K protein, for the hierarchical assembly of the box C/D snoRNP. *Mol. Cell Biol.* 22, 8342–8352.
- Weiske, J., and Huber, O. (2005). The histidine triad protein Hint1 interacts with Pontin and Reptin and inhibits TCF-beta-catenin-mediated transcription. *J. Cell Sci.* 118, 3117–3129.
- Wiese, C., Wilde, A., Moore, M. S., Adam, S. A., Merdes, A., and Zheng, Y. (2001). Role of importin-beta in coupling Ran to downstream targets in microtubule assembly. *Science* 291, 653–656.
- Wilde, A., and Zheng, Y. (1999). Stimulation of microtubule aster formation and spindle assembly by the small GTPase Ran. *Science* 284, 1359–1362.
- Wong, J. *et al.* (2007). A protein interaction map of the mitotic spindle. *Mol. Biol. Cell* 18, 3800–3809.
- Xu, Z. X., Zou, W. X., Lin, P., and Chang, K. S. (2005). A role for PML3 in centrosome duplication and genome stability. *Mol. Cell* 17, 721–732.
- Xue, Y., Canman, J. C., Lee, C. S., Nie, Z., Yang, D., Moreno, G. T., Young, M. K., Salmon, E. D., and Wang, W. (2000). The human SWI/SNF-B chromatin-remodeling complex is related to yeast rsc and localizes at kinetochores of mitotic chromosomes. *Proc. Natl. Acad. Sci. USA* 97, 13015–13020.
- Zhang, C., Hughes, M., and Clarke, P. R. (1999). Ran-GTP stabilises microtubule asters and inhibits nuclear assembly in *Xenopus* egg extracts. *J. Cell Sci.* 112, 2453–2461.
- Zhang, L., Keating, T. J., Wilde, A., Borisy, G. G., and Zheng, Y. (2000). The role of Xgrip210 in gamma-tubulin ring complex assembly and centrosome recruitment. *J. Cell Biol.* 151, 1525–1536.
- Zheng, Y., Wong, M. L., Alberts, B., and Mitchison, T. (1995). Nucleation of microtubule assembly by a gamma-tubulin-containing ring complex. *Nature* 378, 578–583.