

Microtubule Cytoskeleton Remodeling by Acentriolar Microtubule-organizing Centers at the Entry and Exit from Mitosis in *Drosophila* Somatic Cells

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Cytoskeleton microtubules undergo a reversible metamorphosis as cells enter and exit mitosis to build a transient mitotic spindle required for chromosome segregation. Centrosomes play a dominant but dispensable role in microtubule (MT) organization throughout the animal cell cycle, supporting the existence of concurrent mechanisms that remain unclear. Here we investigated MT organization at the entry and exit from mitosis, after perturbation of centriole function in *Drosophila* S2 cells. We found that several MTs originate from centriolar microtubule-organizing centers (aMTOCs) that contain γ -tubulin and require Centrosomin (Cnn) for normal architecture and function. During spindle assembly, aMTOCs associated with peripheral MTs are recruited to centriolar spindle poles by an Ncd/dynein-dependent clustering mechanism to form rudimentary aster-like structures. At anaphase onset, down-regulation of CDK1 triggers massive formation of cytoplasmic MTs de novo, many of which nucleated directly from aMTOCs. CDK1 down-regulation at anaphase coordinates the activity of Msp/XMAP215 and the kinesin-13 KLP10A to favor net MT growth and stability from aMTOCs. Finally, we show that microtubule nucleation from aMTOCs also occurs in cells containing centrosomes. Our data reveal a new form of cell cycle-regulated MTOCs that contribute for MT cytoskeleton remodeling during mitotic spindle assembly/disassembly in animal somatic cells, independently of centrioles.

INTRODUCTION

In animal somatic cells the centrosome is commonly viewed as the major microtubule-organizing center (MTOC) responsible for the architecture of the MT cytoskeleton throughout the cell cycle and plays a central role during spindle assembly as cells enter mitosis. The centrosome is organized as a pair of centrioles surrounded by a pericentriolar material (PCM) where most MTs nucleate. One of the key components of the PCM is the highly conserved protein γ -tubulin, which has been found in association with other proteins that compose the γ -tubulin ring complex (γ -TuRC). During mitosis in humans and *Drosophila*, this complex is enriched at the centrosome by auxiliary proteins like pericentrin/D-PLP or Cnn and was proposed to play a critical role in MT nucleation (Raynaud-Messina and Merdes, 2007).

Despite the role of the centrosome as MTOC is well established, several noncentrosomal MTs and MTOCs have been found in a wide range of organisms and cell types, including those that normally have a functional centrosome (Spiegelman *et al.*, 1979; Bre *et al.*, 1987; Khodjakov *et al.*, 2000; Sawin and Tran, 2006; Rogers *et al.*, 2008). Early investigations about the origin of these MTs in animal cells revealed that some appear de novo in the cytoplasm (Vorobjev *et al.*, 1997; Yvon and Wadsworth, 1997) and show normal dynamic instability at the plus ends, whereas minus ends

are fairly stable. These findings provided the fundamental premises for free MT formation in the cytoplasm and implied the existence of yet poorly characterized “factors” that participate in the assembly process.

The most profound transformation of the MT cytoskeleton during the cell cycle occurs when cells enter mitosis. At this stage, the proportion of MT polymer abruptly decreases, concomitantly with an increase in MT dynamics (Belmont *et al.*, 1990; Zhai *et al.*, 1996). In vitro studies have shown that this correlates with the activation of CDK1 (Verde *et al.*, 1990), which phosphorylates a large number of microtubule-associated proteins (MAPs), thereby favoring the “search-and-capture” of chromosomes by centrosomal MTs and contributing to mitotic spindle morphogenesis (Kirschner and Mitchison, 1986). Higher plants and several female meiotic systems however, build up a microtubular spindle without the contribution of canonical centrioles, supporting the existence of one or more centriolar spindle assembly pathways (Lloyd and Chan, 2006). One of these pathways has been particularly well characterized using *Xenopus* oocyte extracts in vitro. In this system, spindles form “inside-out” after initial MT nucleation in the vicinity of chromosomes, in a process involving a RanGTP gradient and several molecular motors (Karsenti and Vernos, 2001). Interestingly, centriolar spindle assembly appears to be a conserved process in *Drosophila* and mammalian somatic cells (Debec *et al.*, 1982; Khodjakov *et al.*, 2000) and seems sufficient to drive the development of adult flies from late embryonic stages (Basto *et al.*, 2006). Yet, the underlying molecular and structural requirements behind centriolar spindle formation in animal somatic cells remain largely unknown.

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An even more mysterious and equally extraordinary transformation occurs as mitotic spindles disassemble and the interphase MT cytoskeleton is reestablished when cells exit mitosis. On entering anaphase, CDK1 activity drops as a result of cyclin B degradation by the anaphase-promoting complex (APC) and a balance with the activity of protein phosphatases regulates MT dynamics, favoring their stabilization (Tournebise *et al.*, 1997). In fission yeast this process has been shown to involve nonspindle pole body (the yeast centrosome equivalent) MTOCs, which nucleate MTs from the equatorial site at the end of mitosis and from multiple sites near the cell nucleus during interphase (Sawin and Tran, 2006). More recently, the AAA-ATPase Cdc48/p97 and its adapter Ufd1-Npl4, were implicated in mitotic spindle disassembly in *Saccharomyces cerevisiae* and *Xenopus* oocyte extracts and proposed to regulate several spindle assembly factors like XMAP215, TPX2, and Plx1 (Cao *et al.*, 2003). Overall, these data suggest that MT cytoskeleton rearrangements during mitotic exit involve both cell cycle-dependent regulation of MT dynamics and acentriolar MT nucleation, two features that remain to be demonstrated in animal somatic cells.

To understand the molecular and structural requirements involved in MT cytoskeleton remodeling at the entry and exit from mitosis here, we investigated the impact of the absence of centrioles in spindle assembly and disassembly and the respective role of γ -tubulin in MT nucleation in *Drosophila* somatic cells.

MATERIALS AND METHODS

Transmission Electron Microscopy

Transmission electron microscopy in *Drosophila* S2 and 1182-4D cells was performed as previously described (Debec *et al.*, 1982; Maiato *et al.*, 2002).

Cell Culture and RNA Interference

Drosophila S2 cells and the derivative clone expressing γ -tubulin-green fluorescent protein (GFP) and mCherry- α -tubulin (gift of G. Goshima, Nagoya University, Japan) were cultured as previously described (Maiato *et al.*, 2002). 1182-4D cells were grown at 25°C, with a 1:1 mixture of D22 (United States Biological, Swampscott, MA) and M3 media (Sigma, St. Louis, MO) with 10% fetal bovine serum (FBS). RNA interference (RNAi) was performed as described previously using published targeting sequences (Maiato *et al.*, 2002; Verollet *et al.*, 2006; Goshima *et al.*, 2007; Rodrigues-Martins *et al.*, 2007). For DSas-4 RNAi, cells were harvested after 6 d (for immunofluorescence analysis) or over two to four transfection cycles with 4-d interval for live imaging (as indicated in the text) also as previously described (Rodrigues-Martins *et al.*, 2007). For codepletion experiments, cells were transfected at the time of the last pulse of DSas-4 RNAi (during 4 d), except in the case of EB1 and Msps where cells were transfected at the time of the last two pulses of DSas-4 RNAi (3 + 3 d). Primary antibodies used for Western blot analysis were mouse anti- γ -tubulin (GTU-88 clone, Sigma), anti- α -tubulin (DM1A, Abcam, Cambridge, MA), anti-dynein heavy chain (gift from T. Hays, University of Minneapolis, MN), rabbit anti-Ncd (gift from J. Scholey, UC Davis, CA), anti-Cenp-C (gift from C. Lehner, University of Zurich, Switzerland), anti-Cnn (gift from T. Megraw, UT Southwestern Medical Center, TX), anti-Dgt5 (gift from G. Goshima, Nagoya University, Japan), anti-KLP10A (gift from D. Sharp, Albert Einstein College of Medicine, NY), anti-Msps (gift from H. Ohkura, University of Edinburgh, UNITED KINGDOM), anti-EB1 (gift from R. Vale, UCSF, CA), and anti-D-CLIP-190 (gift from K. Miller, Washington University, MO). Appropriate secondary antibodies were detected using enhanced chemiluminescence (Pierce, Rockford, IL). Mast and Dgp71WD depletion was assessed by phenotypic analysis (Maiato *et al.*, 2002; Verollet *et al.*, 2006).

Immunofluorescence Microscopy

Cells were placed on 0.5 mg/ml concanavalin A (Sigma)-coated coverslips and fixed after 2 h with 6.4% paraformaldehyde in Cytoskeleton buffer (CB; Maiato *et al.*, 2002) at room temperature or with methanol at -20°C for 10 min, permeabilized with 0.5% SDS in PBS for 3 min (when GTU-88 antibody was used), or extracted with 0.5% Triton X-100 in CB for 10 min and incubated overnight at 4°C with appropriate antibodies. The following primary antibodies were used: rat anti- α -tubulin (YOL1/34, Abcam; or mouse clone B512, Sigma), mouse anti- γ -tubulin (GTU-88, Sigma; or rabbit R62; Debec *et al.*, 1995), rabbit D-PLP and DSas-4 (gifts from J. Raff, Cambridge University, UNITED KINGDOM), Dgp71WD and Dgrip84 (gifts from B. Raynaud-

Messina, Institut de Sciences et Technologies du Médicament de Toulouse, France), and guinea-pig Cnn (1:2000; gift from T. Megraw, UT Southwestern Medical Center, TX). Secondary antibodies used were as follows: Alexa 488, 568, and 647 (Invitrogen, Molecular Probes, Eugene, OR) and DAPI (1 μ g/ml). Three-dimensional data sets of representative cells were collected with a Zeiss Imager.Z1 microscope (Carl Zeiss, Jena, Germany) equipped with an AxiocamMRm CCD, using a 100 \times 1.4 NA oil immersion objective. The entire system was driven by Axiovision software. Images were subsequently blind-deconvolved with AutoDeblur X2 software (Media Cybernetics, Silver Spring, MD) and processed for publication using Adobe Photoshop CS3 and Illustrator 8.0 (Adobe Systems, San Jose, CA). For the induction of MT aster formation, cells were incubated with 10 μ M taxol (Sigma) for 20 min. For quantification of DSas-4 and γ -tubulin localization in centriolar and acentriolar cells in the absence of MTs, 30 μ M colchicine (Sigma) was used for 16 h.

Microtubule Depolymerization/Repolymerization Assay

MTs were depolymerized at 4°C for 2 h and allowed to repolymerize at room temperature for 2 min (see Figure 2) or at different time points (Supplementary Figure S4). Cells were then immediately fixed with methanol at -20°C and processed for immunofluorescence as described. In the case of Figure 2, representative image galleries were collected, and the areas of 50 asters (α -tubulin) and respective γ -tubulin foci were measured using the Freehand tool of ImageJ 1.38x software (<http://rsb.info.nih.gov/ij/>; NIH, Bethesda, MA) and plotted using Windows Visio Software (Microsoft, Redmond, WA). Only α -tubulin staining in the asters was considered.

Live-Cell Imaging

S2 cells stably expressing γ -tubulin-GFP and mCherry- α -tubulin were plated in 0.25 mg/ml concanavalin A-coated 22 \times 22-mm coverslips and mounted in Rose chambers. Four-dimensional data sets were collected with an Andor Revolution Spinning Disk confocal system (Andor Technology, Belfast, United Kingdom) equipped with an Electron Multiplying CCD iXonEM+ camera and a Yokogawa CSU-22 U (Yokogawa Electric, Tokyo, Japan) based on an Olympus IX81 inverted microscope (Tokyo, Japan). Two laser lines (488 and 561 nm) were used for near-simultaneous excitation of GFP and mCherry, and the system was driven by Andor IQ software. Time-lapse image stacks were collected every 20 or 30 s with 0.5- μ m z-steps and projected as maximum pixel intensities. For MT stabilization, medium was supplemented with 10 μ M taxol (Sigma), respectively.

CDK1 Inhibition Assay

Movies of four cells were analyzed for each RNAi condition. The number of aMTOCs present in each cell was determined before the addition of 50 μ M roscovitine (Sigma) and after proteasome inhibition with 20 μ M MG132 (Sigma) for 2 h. On addition of roscovitine, MT elongation from aMTOCs and the respective stability was compared with DSas-4 RNAi cells.

RESULTS

Characterization of Acentriolar Spindle Poles in *Drosophila* 1182-4D Cells

The *Drosophila* 1182-4D cell line was established from lethal haploid embryos laid by homozygous females for the mh 1182 mutation after spontaneous diploidization under sub-optimal culture conditions (Debec *et al.*, 1982). Together with its original haploid line, they remain the only animal somatic cell lines ever obtained that constitutively lack centrioles, a remarkable feature that has been firmly established by two extensive ultrastructural studies, including high-voltage electron microscopy (EM) from thick sections (Debec *et al.*, 1982; Szollosi *et al.*, 1986). The mh 1182 genetic region does not contain any predicted gene encoding for a centriolar protein (Benjamin Loppin, personal communication). Additionally, 1182-4D cells have been kept in culture for several years and express normal levels of SAK/Plk4 (our unpublished observations), a protein required for de novo centriole formation (Rodrigues-Martins *et al.*, 2007), suggesting that this pathway is maintained inactive by a yet unknown mechanism. Although the acentriolar nature of the 1182-4D cell line remains inexplicable, an intriguing property of these cells is their ability to recruit γ -tubulin to some acentriolar spindle poles during mitosis (Debec *et al.*, 1995).

To shed light on the mechanism of spindle MT organization in animal somatic cells lacking centrioles, we investi-

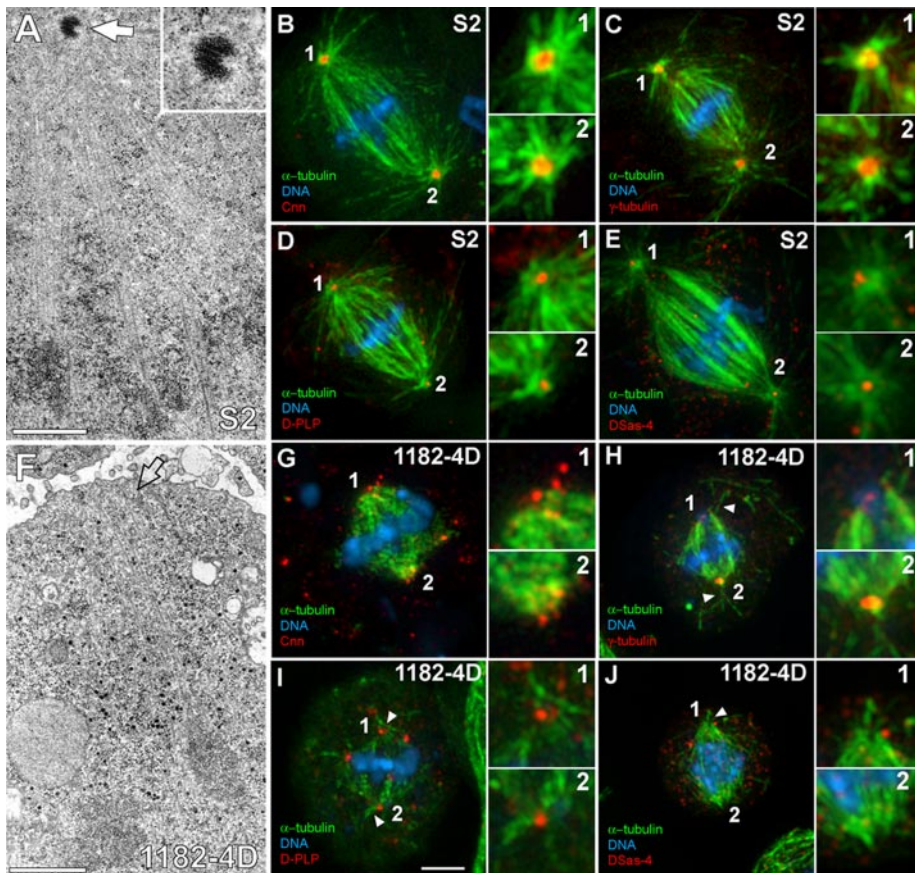


Figure 1. Molecular and structural characterization of acentriolar spindle poles in the 1182-4D cell line. (A) EM picture from a serially sectioned S2 cell containing centrioles (arrow). Inset, higher magnification of the centriole. (B–E) Immunofluorescence showing Cnn (B), γ -tubulin (C), D-PLP (D), and DSas-4 (E) localization at spindle poles of S2 cells. (F) EM picture from a serially sectioned 1182-4D cell showing the lack of centrioles (arrow indicates the spindle pole). (G–J) Immunofluorescence showing Cnn (G), γ -tubulin (H), D-PLP (I), and DSas-4 (J) localization at acentriolar spindle poles of 1182-4D cells. DSas-4 was found accumulated at the centrosomal core in S2 cells but was usually dispersed in the cytoplasm of 1182-4D cells (E and J). Note the presence of few astral MTs associated with acentriolar poles (arrowhead in H–J). Numbered insets correspond to enlarged views of the respective spindle poles. Green, MTs; blue, DNA. Scale bar, 1 μ m for EM and 5 μ m for immunofluorescence pictures.

gated the localization of several centrosomal markers at spindle poles of 1182-4D cells. *Drosophila* S2 cells were used as a control cell line that contains centrioles (Figure 1A). As expected, S2 cells recruited γ -tubulin and Cnn to the PCM and formed robust astral MTs (Figure 1, B and C). Additionally, D-PLP and DSas-4, two bona fide pancentriolar markers (Martinez-Campos *et al.*, 2004; Basto *et al.*, 2006) localized within more restricted areas of the S2 cell mitotic asters that likely represent centrioles (Figure 1, D and E). In 1182-4D cells (Figure 1F) we observed that Cnn and γ -tubulin localization at the poles was disorganized and distinct from S2 cells (Figure 1, G and H). In agreement with previous observations (Debec *et al.*, 1995) we found that approximately one-third of 1182-4D cells showed well-defined γ -tubulin foci in both poles, another third showed focused γ -tubulin in just one pole, whereas the remaining third showed dispersed or undetectable γ -tubulin at both poles (Supplementary Table S1). Noteworthy, a few astral MTs could be detected at the polar regions of 1182-4D cells (Figure 1, H–J). We next evaluated the status of D-PLP and DSas-4 at acentriolar poles of 1182-4D cells. Both proteins were dispersed or eventually formed small aggregates at spindle poles (Figure 1, I and J). Quantification of the polar distribution of DSas-4 in 1182-4D cells indicated that it was undetectable or highly dispersed at the poles in nearly two-thirds of the cells (Supplementary Table S1). Curiously, approximately one-third of 1182-4D cells showed a conspicuous DSas-4 signal in one pole only, whereas a minority of the cells (5%) were able to recruit DSas-4 to both spindle poles, as opposed to \sim 90% of S2 cells (Supplementary Table S1). These results may be explained by the fact that a significant rapidly exchanging fraction of DSas-4 and D-PLP is associated with the PCM

(Martinez-Campos *et al.*, 2004; Dammermann *et al.*, 2008). Overall, these data indicate that although centrioles enhance the organization of PCM proteins at mitotic spindle poles, they are not absolutely required for this process. Furthermore, they support that acentriolar spindle poles that are able to recruit PCM components retain some MT nucleation capacity.

To quantify the capacity that γ -tubulin-positive spindle poles in 1182-4D cells have to organize MT asters, we performed a MT depolymerization/repolymerization assay and measured the area of the resulting asters (Figure 2A). We have found that the area occupied by the asters in 1182-4D cells was 3–4-fold smaller than in S2 cells (Figure 2, B and C, and B' and C'), which correlated well with the areas of the respective γ -tubulin foci (Figure 2D). This suggests that centrioles normally determine the amount of γ -tubulin recruited, thereby regulating the size of the asters, as has been shown in other systems (Kirkham *et al.*, 2003). Altogether, these experiments indicate that acentriolar spindle poles in animal somatic cells retain some capacity to recruit γ -tubulin and form rudimentary MT asters.

An Ncd/Dynein-dependent Microtubule Clustering Mechanism Recruits γ -Tubulin to Acentriolar Spindle Poles

1182-4D cells are two- to threefold smaller than S2 cells and assemble a short spindle (typically around 5 μ m long), which makes them highly unfavorable for high-resolution microscopy studies. To circumvent this problem and investigate the mechanism of γ -tubulin recruitment to acentriolar spindle poles in living cells, we knocked down DSas-4 by RNAi in S2 cells. DSas-4 is a conserved protein required for centriole duplication, and after several cell cycles with reduced levels of DSas-4, cells are gradually born without

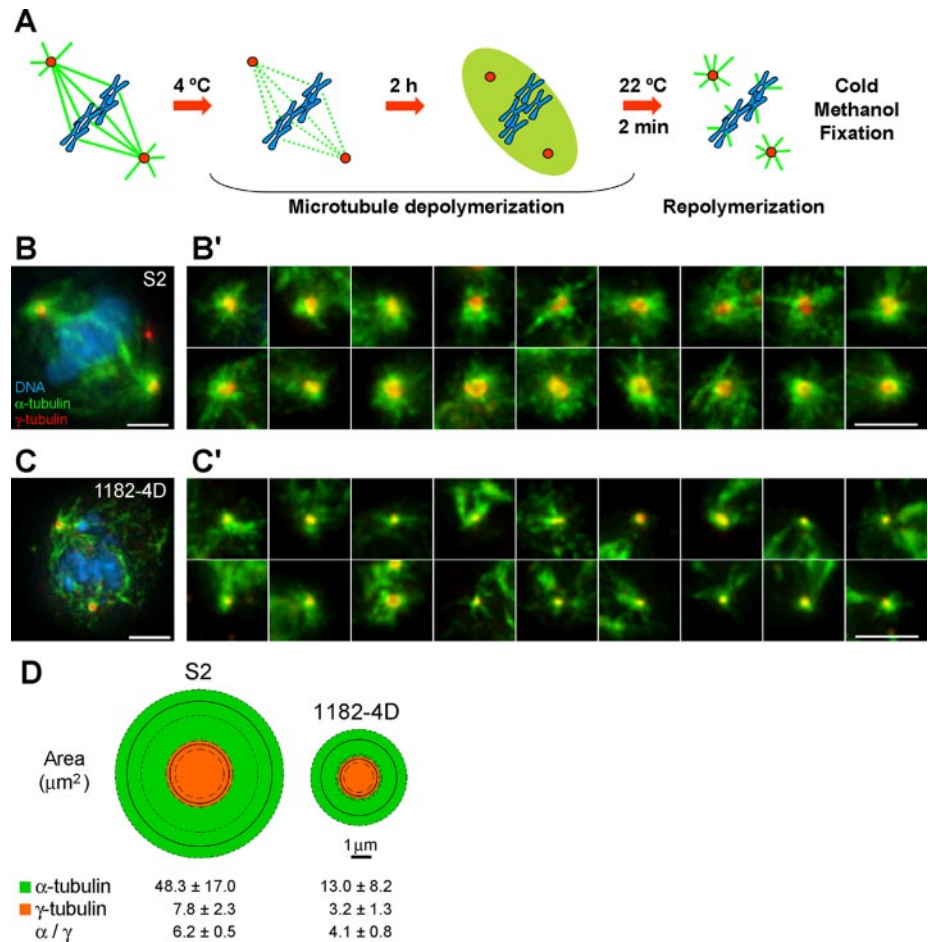


Figure 2. Microtubule organization from acentriolar poles containing γ -tubulin. (A) MT depolymerization/repolymerization assay. (B and C) Localization of γ -tubulin (red) in S2 (B) and 1182-4D (C) cells after MT repolymerization and respective image galleries of MT asters obtained from γ -tubulin-positive poles in S2 (B') and 1182-4D cells (C'). Green, MTs; blue, DNA. Scale bar, (B and C) 5 μ m; (B' and C') 2 μ m. (D) Graphic representation of the areas corresponding to the aster (green) and respective γ -tubulin (orange) recruited in S2 and 1182-4D cells. Solid and dashed lines, respectively, represent the average and SD of the sample ($n = 50$ asters for each cell line).

centrioles (Basto *et al.*, 2006; Rodrigues-Martins *et al.*, 2007). As so, we performed several rounds of DSas-4 depletion by RNAi in S2 cells. Because DSas-4 is not an abundant protein in *Drosophila* cells, we were unable to monitor the efficiency of depletion by Western blot (Basto *et al.*, 2006; Rodrigues-Martins *et al.*, 2007). However, the presence or absence of DSas-4 at spindle poles could easily be inferred by immunofluorescence with anti-DSas-4 antibodies. In control S2 cells DSas-4 was always detected as well-defined spots in the center of γ -tubulin-containing asters (Supplementary Figure S1A), whereas after only two rounds of DSas-4 RNAi over 6–7 d, DSas-4 could not be detected at spindle poles in the vast majority of the cells (Supplementary Figure S1, B–E). As in 1182-4D cells, γ -tubulin recruitment to DSas-4-negative poles still occurred to some extent (either in the form of focused or dispersed aggregates) in nearly two-thirds of the cells (Supplementary Figure S1, B–F). Similar results were obtained after SAK or DSas-6 RNAi (our unpublished observations). We concluded that as in the case of 1182-4D cells, S2 cells retain some capacity to recruit γ -tubulin to acentriolar spindle poles.

We next used dual-wavelength spinning-disk confocal microscopy to follow mitotic spindle assembly in S2 cells stably expressing mCherry- α -tubulin and γ -tubulin-GFP at near endogenous levels (Figure S2A) after DSas-4 RNAi. We found that, after two rounds of DSas-4 RNAi over 6–7 d, DSas-4 characteristic labeling was occasionally present in only one spindle pole (Supplementary Figure S1G), suggesting that these mitotic spindles were composed of one cent-

riolar and one acentriolar pole. As so, we used this feature to compare the respective behavior of γ -tubulin-GFP at each pole within the same cell (Figure 3 and Supplementary Video S1). Although presumably centriolar poles were found associated with prominent MT asters containing a morphologically unaltered focus of γ -tubulin-GFP over time (Figure 3A1 and Supplementary Video S1), acentriolar poles showed highly dynamic γ -tubulin-GFP aggregates associated with spindle MT minus ends (Figure 3A2 and Supplementary Video S1). Some γ -tubulin-GFP aggregates in the cytoplasm appear associated with peripheral MTs nucleated de novo, which were subsequently incorporated to the acentriolar pole by an MT-clustering mechanism, thereby contributing to mitotic spindle morphogenesis (Figure 3A2 and Supplementary Video S1). Similar aggregates of endogenous γ -tubulin could be found by immunofluorescence, which colocalize with γ -TuRC components and Cnn, but not with the Golgi apparatus, throughout mitosis in control and DSas-4-depleted cells (Supplementary Figure S3, and our unpublished observations).

To confirm that the observed cytoplasmic γ -tubulin aggregates are competent to organize MTs, we performed a time-course MT depolymerization/repolymerization assay similar to that described in Figure 2, but over an extended period (Supplementary Figure S4). We observed that endogenous γ -tubulin aggregates in the cytoplasm are resistant to cold-induced MT depolymerization and were present in control or DSas-4-depleted S2 cells (Supplementary Figure S4, A and B). Shortly after rewarming, several cytoplasmic

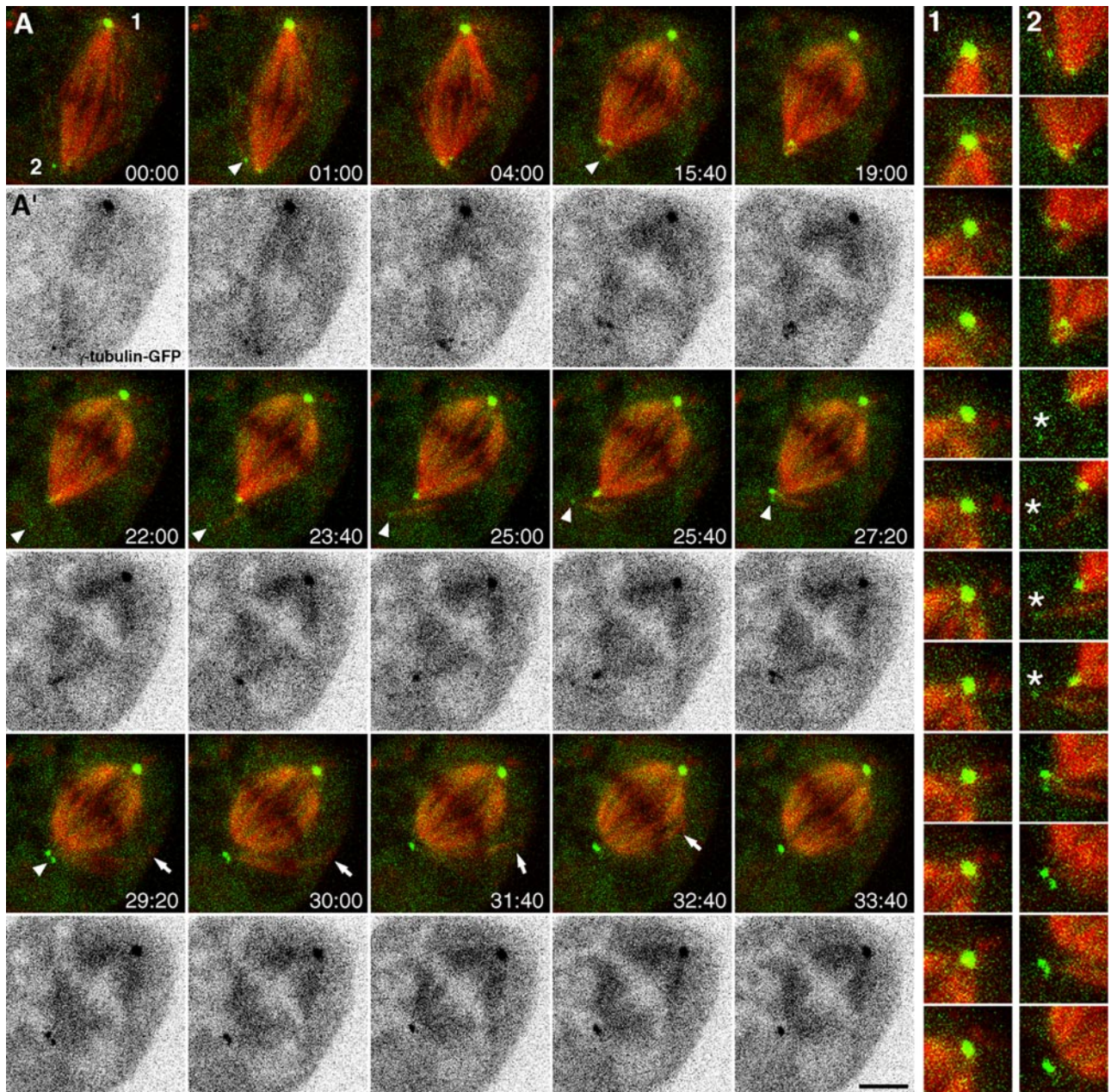


Figure 3. Live-cell imaging of γ -tubulin and spindle microtubules after DSas-4 RNAi in S2 cells. (A) Time-lapse sequence showing mitotic spindle assembly in S2 cells stably expressing mCherry- α -tubulin (red) and γ -tubulin-GFP (green) after DSas-4 RNAi. One centriole-containing pole (A1) and one acentriolar pole (A2) is shown in higher magnification for comparison. Asterisks indicate a small aggregate of γ -tubulin that associates with MTs nucleated de novo, which are subsequently clustered into the spindle pole. Note that γ -tubulin recruited to the acentriolar pole gradually detaches from the MT minus ends. Arrowheads in A highlight small γ -tubulin-GFP aggregates associated with MT minus ends that are clustered into the acentriolar pole. Arrows show the incorporation of peripheral MTs associated with γ -tubulin aggregates into the mitotic spindle. (A') Same time-lapse sequence depicting γ -tubulin-GFP after contrast inversion. Time is in min:sec; scale bar, 5 μ m.

MTs appear to nucleate from γ -tubulin aggregates, regardless of the presence of centrosomes (Supplementary Figure S4, A' and B'). We named these γ -tubulin aggregates associated with cytoplasmic MTs as aMTOCs. Noteworthy, the relative abundance of MTs associated with aMTOCs decreases over time as spindles assemble after rewarming, suggesting that some of those MTs are either incorporated into the spindle or are unstable (Supplementary Figure S4, A'' and B'').

In DSas-4-depleted cells, γ -tubulin-GFP aggregates initially concentrate in a region with high MT density sur-

rounding the chromosomes and some are dispersed in the cytoplasmic periphery (Figure 4, A and A'). As spindles start to form "inside-out," these aggregates concentrate at acentriolar poles containing focused MT minus ends and remain there in a relatively well-organized manner until anaphase onset when they start to disperse. This behavior is consistent with the involvement of MT minus-end-directed motors with cross-linking activity. Therefore we investigated how codepletion of Ncd or cytoplasmic dynein with DSas-4 impairs the recruitment of γ -tubulin to acentriolar poles (Fig-

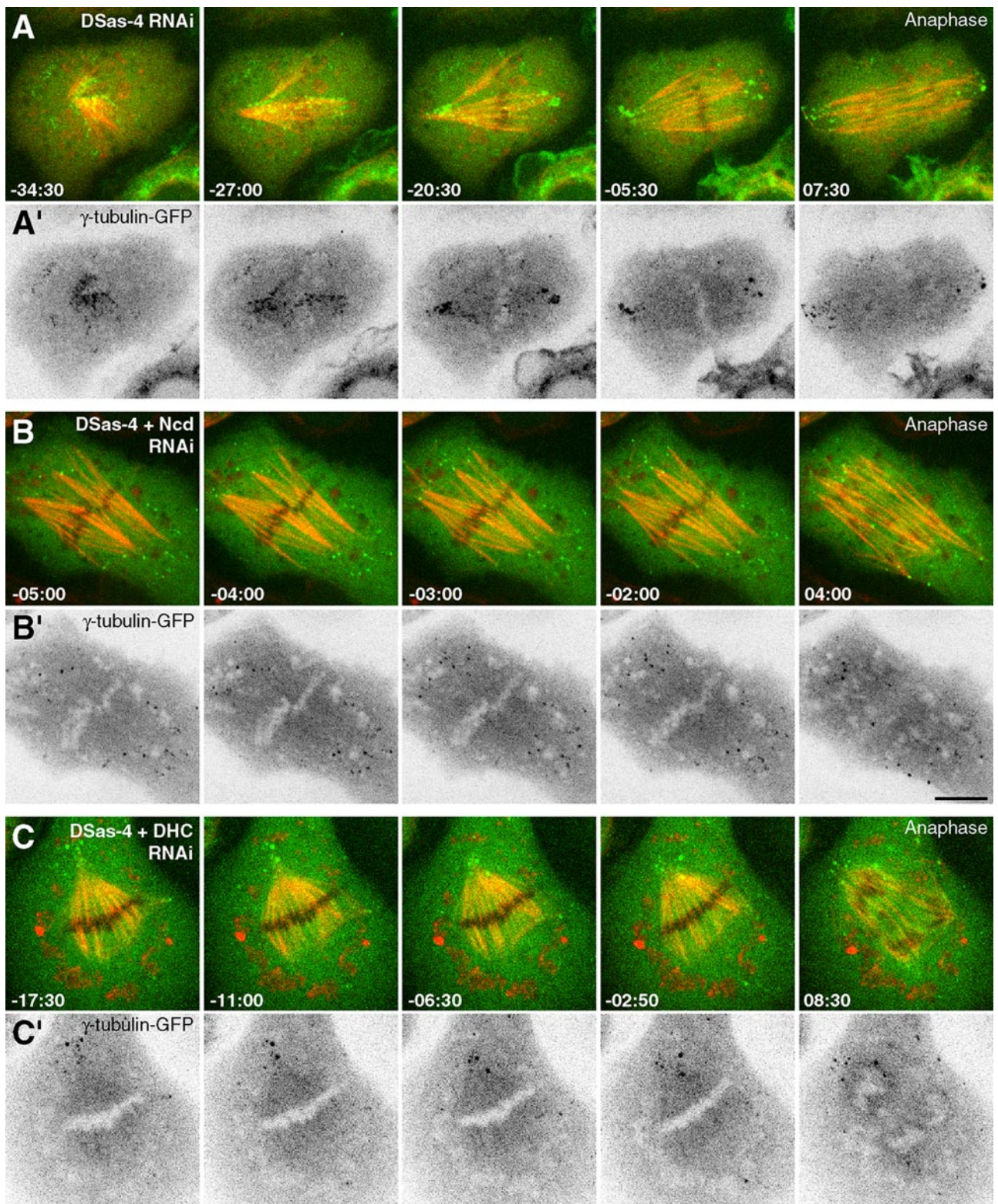


Figure 4. Ncd/dynein-dependent microtubule clustering mechanism recruits γ -tubulin to acentriolar spindle poles. (A–C) Time-lapse sequences showing mitotic spindle assembly in S2 cells stably expressing mCherry- α -tubulin (red) and γ -tubulin-GFP (green) after DSas-4 RNAi. (A'–C') Corresponding time-lapse sequences depicting γ -tubulin-GFP after contrast inversion. (A and A') Spindle assembly in an S2 cell after DSas-4 RNAi showing γ -tubulin aggregates moving poleward and remaining clustered at acentriolar spindle poles. (B and B') Spindle assembly in a cell codepleted for DSas-4 and Ncd. (C and C') Spindle assembly in a cell codepleted for DSas-4 and DHC. Time is in min:sec; scale bar, 5 μ m. Time zero was set from the moment of anaphase onset.

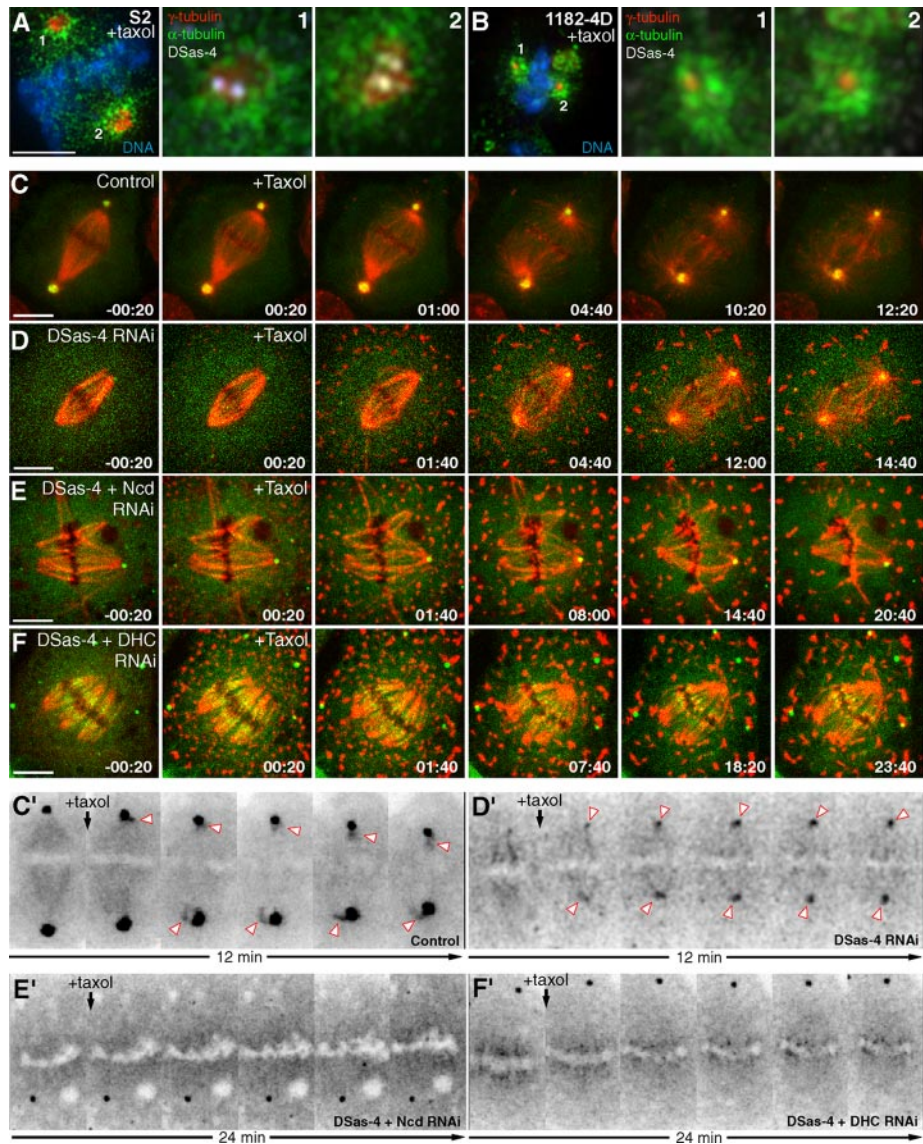


Figure 5. γ -Tubulin recruitment into taxol-induced acentriolar microtubule asters requires Ncd and dynein. (A and B) Immunofluorescence of S2 (A) and 1182-4D cells (B) after taxol treatment showing the distribution of endogenous γ -tubulin (red) and DSas-4 (white). Numbered pictures correspond to enlarged views of the respective spindle poles in A and B. MTs are in green and DNA in blue. (C–F) Time-lapse sequences showing γ -tubulin redistribution upon taxol-induced aster formation in control, DSas-4 RNAi, DSas-4+Ncd RNAi, DSas-4+DHC RNAi, respectively, in S2 cells stably expressing mCherry- α -tubulin (red) and γ -tubulin-GFP (green). (C'–F') Corresponding contrast inversion views. After codepletion of DSas-4 with Ncd or DHC, aster formation after taxol treatment is strongly compromised and γ -tubulin is no longer recruited to acentriolar spindle poles. Time zero was set from the moment of taxol addition. Time lapse is in min:sec; scale bars, 5 μ m.

ure 4, Supplementary Videos S2–S4). As previously shown (Goshima *et al.*, 2005; Morales-Mulia and Scholey, 2005), Ncd is required to maintain MT minus ends clustered, which we found to contribute to focus γ -tubulin-GFP aggregates at acentriolar poles (Figure 4, B and B'). Dynein also enhances this process, but its role appears to be less important than Ncd, as acentriolar poles in 25% of the depleted cells retain MT-clustering capacity with few associated γ -tubulin-GFP aggregates (Figure 4, C and C').

γ -Tubulin Recruitment into Acentriolar Poles after Taxol-induced Aster Formation Requires Ncd and Dynein

Aster formation induced by taxol in acentriolar *Xenopus* oocyte extracts *in vitro* rely on the motor activity of cyto-

plasmic dynein, which is also necessary to recruit γ -tubulin (Verde *et al.*, 1991; Stearns and Kirschner, 1994). The mammalian orthologue of Ncd, HSET, has also been shown to be specifically required for taxol-induced aster formation both in cultured cells and cell-free extracts (Mountain *et al.*, 1999), but whether it plays a role in recruiting γ -tubulin is not known. Treatment of S2 or acentriolar 1182-4D cells with taxol leads to the formation of asters containing γ -tubulin, regardless of the presence of centrioles (Figure 5A, 1 and 2, and B, 1 and 2). Because spindle MTs are recruited to the aster after taxol addition in control cells, spindle-associated γ -tubulin-GFP starts to concentrate at the MT minus ends close to the centrosome (Figure 5, C and C'). In DSas-4-depleted S2 cells treated with taxol the transport of spindle-

associated γ -tubulin-GFP to the center of the acentriolar aster (Figure 5, D and D') was found to rely on the activity of Ncd and dynein (Figure 5, E and E', and F and F'). These experiments reveal that, in addition to MT clustering of γ -tubulin aggregates in the cytoplasm, MT minus-end-directed transport may also contribute to recruit γ -tubulin from the spindle to acentriolar poles.

De Novo Microtubule Nucleation from aMTOCs during Anaphase Is Regulated by CDK1

After spindle formation many aMTOCs are not found associated with MTs (Figure 6A and Supplementary Videos S5 and S6). To test whether MT nucleation from aMTOCs is cell cycle-regulated, we followed MTs and γ -tubulin-GFP in living S2 cells after DSas-4 RNAi at the metaphase-anaphase transition (Figure 6A and Supplementary Videos S5 and S6). Remarkably, upon anaphase onset, several MTs abruptly form de novo, many of which nucleated directly from aMTOCs that were already present during metaphase (Figure 6, A and A' and Supplementary Videos S5 and S6).

CDK1 is a key cell cycle regulator whose levels influence MT dynamics and stability in interphase and mitosis in vitro (Verde *et al.*, 1990). As so, we reasoned that MT nucleation from aMTOCs could be regulated by CDK1. To test this hypothesis, we treated control and DSas-4-depleted cells with the selective CDK inhibitor roscovitine (Meijer *et al.*, 1997). To rule out unspecific effects on CDKs other than CDK1, we previously blocked cells in metaphase with the proteasome inhibitor MG132, which prevents sister-chromatid separation in a CDK1-independent manner (Skoufias *et al.*, 2007). Addition of roscovitine to MG132-treated metaphase cells immediately triggers formation of MTs in the cytoplasm, many of which nucleated directly from aMTOCs, regardless of the presence of centrosomes (Figures 6, B and B', and C and C', and 7D, and Supplementary Video S7). Because CDK1 is the main CDK controlling mitotic progression, these results indicate that de novo MT nucleation from aMTOCs during anaphase requires down-regulation of CDK1 and occurs in cells that normally contain centrosomes.

CDK1 Controls the Activity of Microtubule-stabilizing and -destabilizing Proteins Involved in Microtubule Formation from aMTOCs during Anaphase

To identify MAPs that participate in de novo MT formation from aMTOCs during anaphase and are under control of CDK1 activity, we performed the same roscovitine/MG132 assay after codepletion of DSas-4 with candidate MAPs, namely Msps, EB1, D-CLIP-190, Mast/Orbit/CLASP, and the kinesin-13 KLP10A (Figure 7, A and B', and D, and data not shown). We found that RNAi depletion of Msps significantly affected normal MT elongation from aMTOCs (cf. Figures 7, A and A', and B and B', with Figure 6, B and B'), whereas knockdown of KLP10A leads to the formation of longer MTs from aMTOCs after addition of roscovitine. RNAi depletion of EB1, D-CLIP-190 and Mast did not significantly affect de novo MT formation from aMTOCs in our assay (Figure 7D and data not shown). These results support a model where CDK1 inactivation during anaphase coordinates the activity of key MT-stabilizing and -destabilizing proteins, which is essential to mediate MT growth from aMTOCs and regulate MT length.

Cnn Is Required for Normal Architecture and Function of aMTOCs

Acentriolar γ -tubulin aggregates extensively colocalize with Cnn and other γ -TuRC components throughout mitosis,

suggesting that these accessory proteins might be required for the normal architecture and/or function of aMTOCs. To directly test whether Cnn and γ -TuRCs are involved in MT formation from aMTOCs, we reproduced our roscovitine/MG132 assay after codepletion of DSas-4 with Cnn or the γ -TuRC component Dgp71WD. This allowed us to circumvent the problem associated with depletion of γ -TuRCs, which severely affects spindle assembly and function (Verollet *et al.*, 2006). We observed that depletion of Cnn reduces the number and size of cytoplasmic γ -tubulin aggregates in metaphase, before roscovitine addition (Figure 7, C, C', and D). Depletion of Dgp71WD or the Augmin component Dgt5 (Goshima *et al.*, 2008), on the other hand, appears to impair the size of aMTOCs (Figure 7D and data not shown). Importantly, upon addition of roscovitine, only depletion of Cnn significantly impaired the function of aMTOCs and affected the stability of MTs formed de novo (Figure 7, C, C'', and D).

DISCUSSION

Role of aMTOCs in Microtubule Organization during Spindle Assembly

Recruitment and stabilization of PCM proteins in animal somatic cells is thought to be a centriole-dependent function (Kirkham *et al.*, 2003; Martinez-Campos *et al.*, 2004; Basto *et al.*, 2006; Rodrigues-Martins *et al.*, 2007). This view is largely supported by the results presented in our work in the sense that centrosomes work as a platform for the organized assembly of PCM. However, here we report that many MTs in *Drosophila* somatic cells with compromised centriole biogenesis are nucleated de novo from cytoplasmic aMTOCs containing several PCM components and may contribute for the remodeling of the MT cytoskeleton at the entry and exit from mitosis in acentriolar systems. Likewise, higher plants are naturally devoid of centrosomes and are still able to organize complex microtubule networks from multiple sites that recruit γ -tubulin and associated proteins (Liu *et al.*, 1993; Murata *et al.*, 2005). Importantly, the aMTOCs described here are also present in centriole-containing cells, and their contribution to MT organization should not be underestimated given that cytosolic γ -tubulin comprises more than 80% of the total cellular pool (Moudjou *et al.*, 1996). In support of this idea, γ -tubulin has been localized in noncentrosomal cytoplasmic patches of polarized mammalian cells (Reilein *et al.*, 2005) and recently shown to mediate microtubule nucleation from the Golgi apparatus in epithelial cells (Efimov *et al.*, 2007). Our results thus uncover an important evolutionary conservation in MT cytoskeleton organization between centriolar and acentriolar systems. It should be noted, however, that the contribution of acentriolar MT organization in animal somatic cells may vary from cell type to cell type (e.g., fibroblasts vs. epithelial cells; Bre *et al.*, 1987), depending on the relative pressure imposed by centrosomes on the concerted recruitment of γ -tubulin and PCM components to the centrosome.

Acentriolar spindle assembly in *Xenopus* oocyte extracts is mediated by a RanGTP gradient concentrated on chromatin and by the action of microtubule motors (Karsenti and Vernos, 2001). However, acentriolar poles of female mouse meiotic spindles can form in the absence of chromatin and independently of RanGTP by self-assembled MT asters that recruit γ -tubulin (Gueth-Hallonet *et al.*, 1993; Palacios *et al.*, 1993; Brunet *et al.*, 1998; Dumont *et al.*, 2007; Schuh and Ellenberg, 2007). In the case of plants, it has been argued that some possess a pole-based mechanism that mediates acentriolar spindle formation during

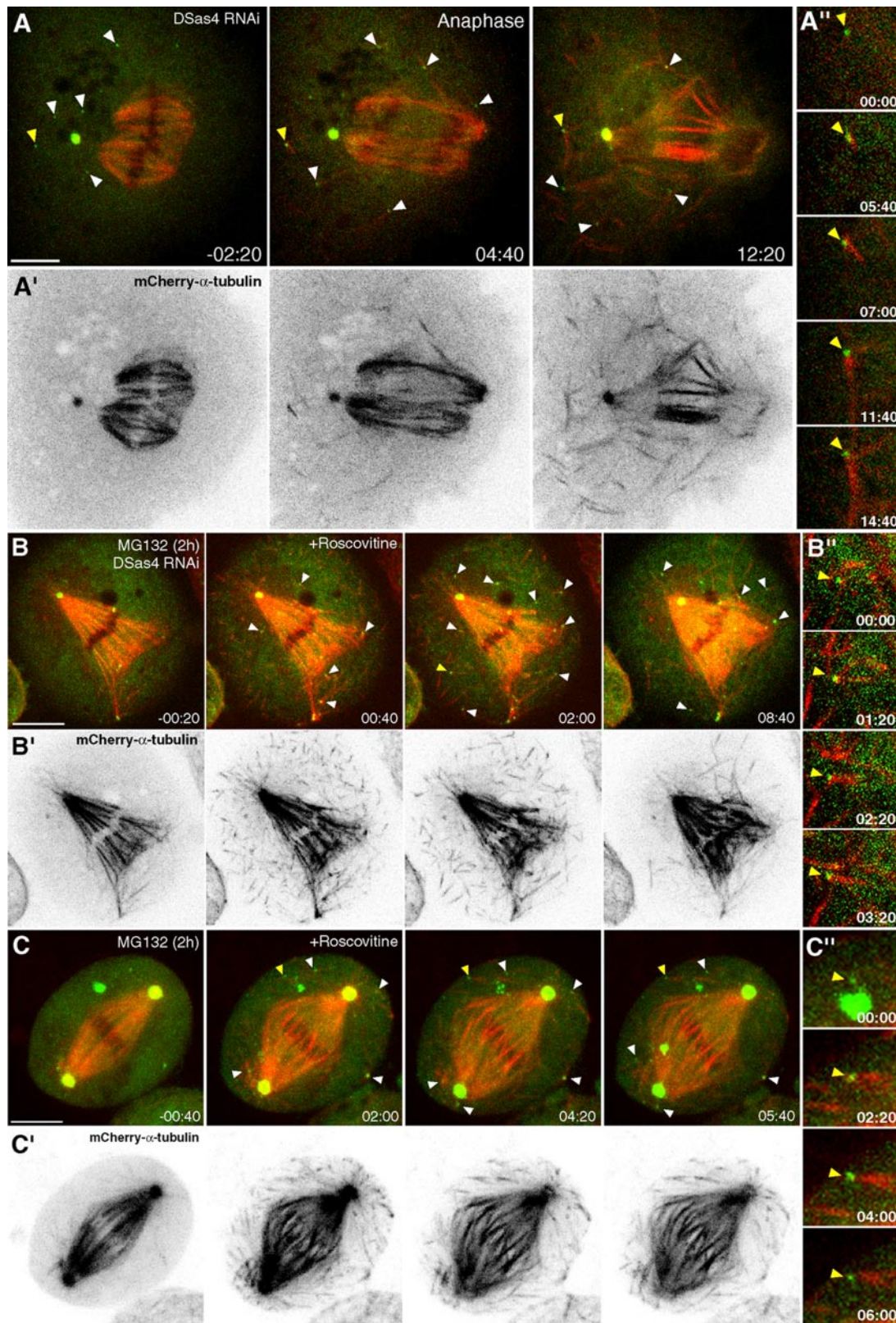


Figure 6. De novo microtubule nucleation from cytoplasmic aMTOCs after anaphase onset is controlled by CDK1. (A–C) Time-lapse sequences of S2 cells stably expressing mCherry- α -tubulin (red) and γ -tubulin-GFP (green) after Dsas-4 RNAi (A and B) or control cells (C). (A'–C') Corresponding time-lapse sequences depicting mCherry- α -tubulin after contrast inversion. (A) After anaphase onset, cytoplasmic aMTOCs (arrowheads) show a sudden formation of MTs while the cytoskeleton is remodeled during mitotic exit. Time zero was set as the time of anaphase onset. (B and C) Dsas-4 RNAi (B and B') and control (C and C') cells, blocked in metaphase for 2 h with MG132 upon addition of roscovitine (time zero). On CDK1 inhibition, cytoplasmic aMTOCs (arrowheads) abruptly organize MTs, regardless of the presence of centrosomes. (A''–C'') Enlarged views of cytoplasmic aMTOCs nucleating MTs de novo (yellow arrowheads in A–C, respectively). Time is in min:sec; scale bars, 5 μ m.

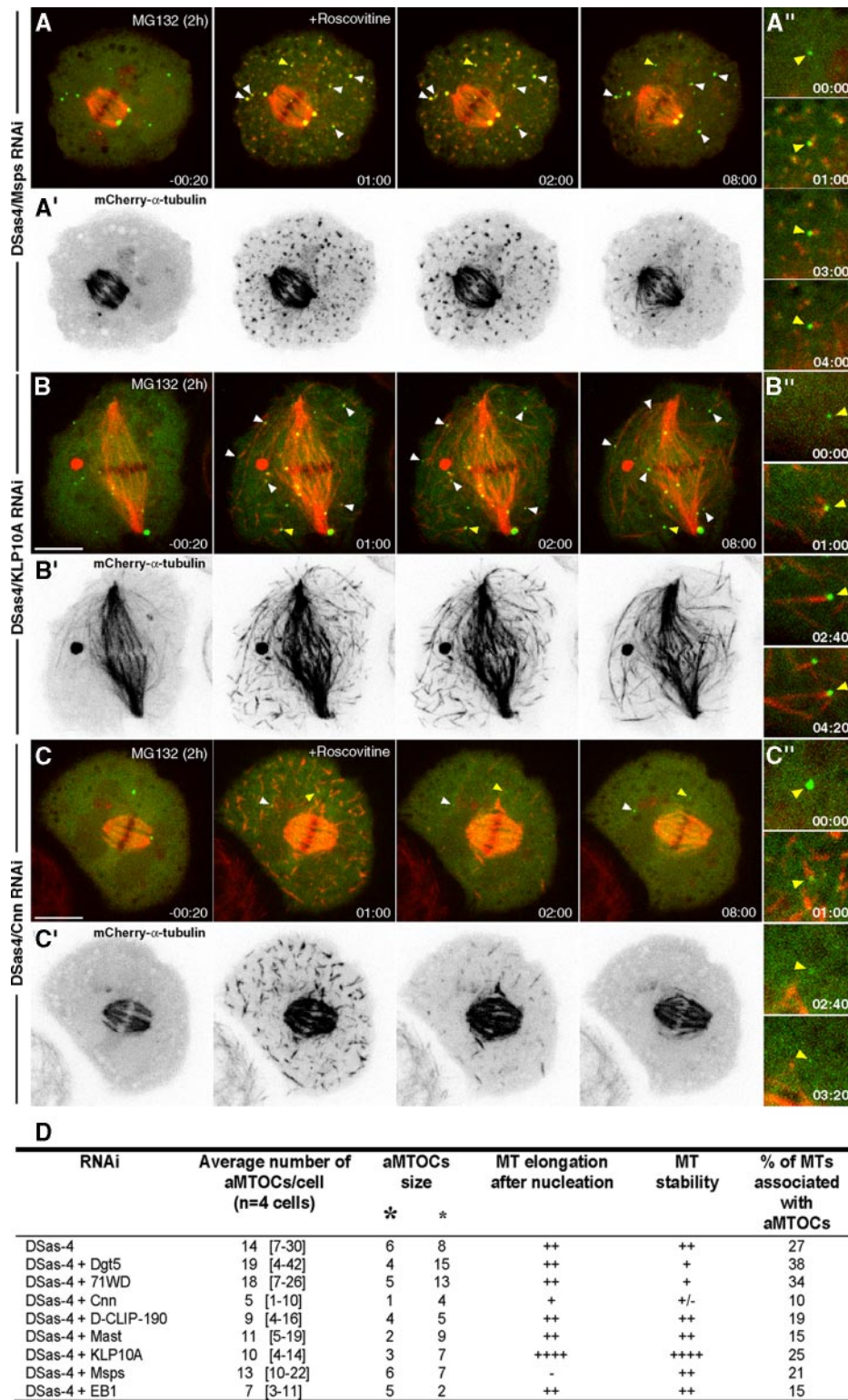


Figure 7. Cnn, Msps and KLP10A regulate the formation and function of aMTOCs. (A–C) Time-lapse sequences of S2 cells stably expressing mCherry- α -tubulin (red) and γ -tubulin-GFP (green) after block in metaphase for 2 h with MG132 and upon addition of roscovitine (time 0). (A'–C') Corresponding time-lapse sequences depicting mCherry- α -tubulin after contrast inversion. (A and A') De novo MT formation after codepletion of DSas-4 and Msps. Note that MTs do not elongate (A'). (B and B') De novo MT formation after codepletion of DSas-4 and KLP10A. Note that MTs are very long and stable (B'). (C and C') De novo MT formation after codepletion of DSas-4 and Cnn. Only a few aMTOCs can be observed and MTs that formed de novo are unstable, disappearing shortly after (C'). (A'–C') Enlarged views of cytoplasmic aMTOCs nucleating MTs de novo (yellow arrowheads in A–C, respectively). Time is in min:sec; scale bars, 5 μ m. (D) Quantification of the number and size of aMTOCs (large and small asterisks represent big and small, respectively), respective MT elongation/stability potential and frequency of de novo nucleated MTs associated with aMTOCs. MT elongation/stability is expressed as a measure relative to DSas-4 RNAi.

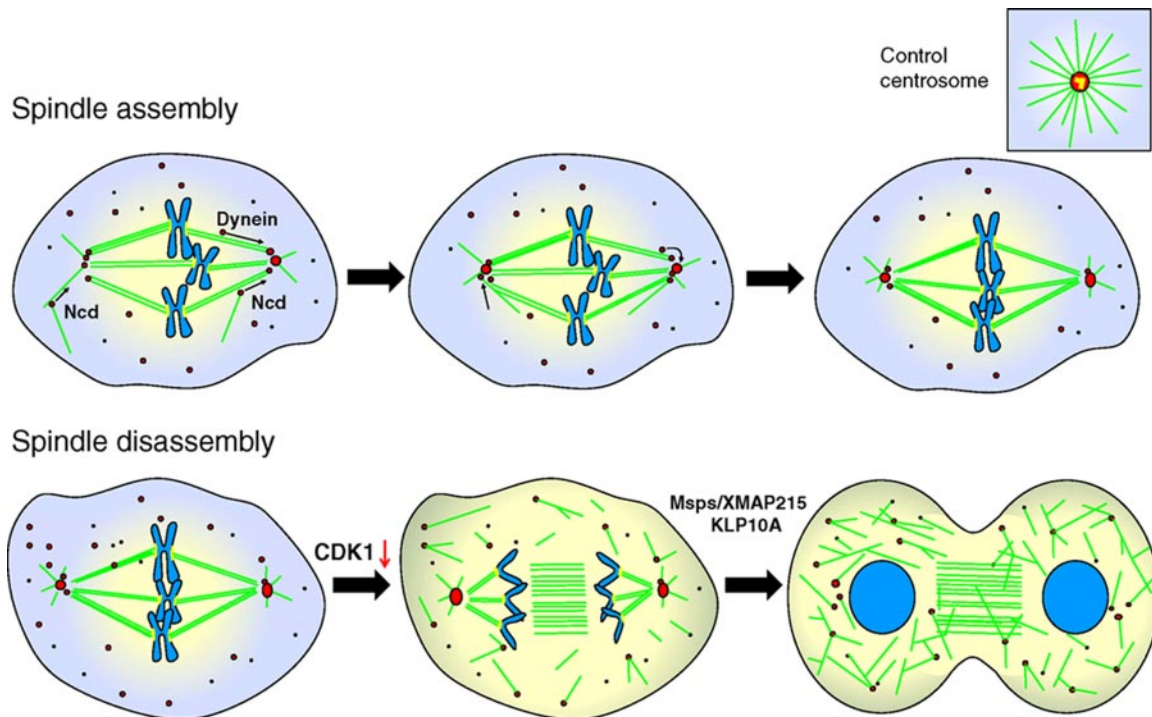


Figure 8. Model for the role of aMTOCs during mitotic spindle assembly and disassembly. Ncd/dynein-dependent mechanism is responsible for recruiting and maintaining γ -tubulin (red circles) in acentriolar poles during mitotic spindle assembly. These poles form rudimentary MT asters when compared with poles containing centrioles, which may work as platforms for the organized recruitment of PCM to the centrosome. De novo MT formation from aMTOCs upon anaphase onset is driven by CDK1 down-regulation, which controls the activity of Msps/XMAP215 and KLP10A to promote net MT growth as cells exit mitosis, thereby contributing to reestablish the interphase MT cytoskeleton.

mitosis (Lloyd and Chan, 2006), where γ -tubulin nucleates spindle MTs from polar caps around the nucleus during prophase (Liu *et al.*, 1993; Brown *et al.*, 2004). We envision that an equivalent mechanism may contribute to spindle morphogenesis in animal somatic cells and work in concert with the centrosome- and/or chromatin-mediated pathways. In fact, EB1-GFP tracking in *Drosophila* S2 cells after centrosome detachment from the spindle revealed new MT nucleation events at acentriolar spindle MT minus ends (Mahoney *et al.*, 2006). The vast majority of EB1 tracks were oriented toward chromosomes but few were found to move away from the pole toward the periphery of the cell. This is consistent with our observation that acentriolar poles that recruit γ -tubulin were able to organize rudimentary astral microtubules. One interesting question arises though—if most cytoplasmic aMTOCs are repressed until anaphase, how would they conceivably contribute to spindle assembly? One possibility would be that additional MT-stabilizing factors allow MT nucleation to take place in the vicinity of the poles or in proximity with unaligned chromosomes. This would also be consistent with the formation of a RanGTP gradient centered on chromosomes that promotes MT nucleation and the existence of factors (e.g., TPX2) involved in this pathway, which are associated with the poles (Wittmann *et al.*, 2000). Another possibility would be that MT nucleation from aMTOCs before anaphase might be regulated by a different balance between the activity of other protein kinases and/or phosphatases, which render MTs to be less stable.

Here we report that an Ncd/dynein-dependent MT clustering mechanism involved in spindle assembly is important to

recruit γ -tubulin associated with peripheral MT minus ends to acentriolar spindle poles (Figure 8). Homozygous Ncd mutants in *Drosophila* are viable but female sterile because of the requirement of Ncd activity for acentriolar spindle assembly in meiotic oocytes (Matthies *et al.*, 1996). It is also known that in mammalian epithelial cells noncentrosome-associated MTs contribute to spindle formation even in the presence of centrosomes, in a dynein-dependent manner (Rusan *et al.*, 2002). Our correlative live-cell imaging of MTs and γ -tubulin supports that γ -tubulin is actively involved in nucleation/stability of MTs from noncentrosomal sites, while participating in spindle formation in animal cells. Whether the contribution of peripheral MTs is essential for spindle formation is unlikely, given the possible redundancy with concurrent centrosome- and/or chromosome-mediated pathways.

Finally, γ -tubulin and γ -TuRCs have recently been demonstrated to play a role in acentrosomal spindle assembly in animal somatic cells (Luders *et al.*, 2006; Mahoney *et al.*, 2006). Noteworthy, γ -TuRCs were dispensable for MT nucleation and to target γ -tubulin to centrosomes, but were critical to recruit γ -tubulin to the spindle (Verollet *et al.*, 2006). More recently a new complex composed of at least five proteins, known as Augmin, also targets γ -tubulin to the spindle and is necessary to increase MT density within the spindle (Goshima *et al.*, 2008). Despite γ -TuRC components were recruited to some cytoplasmic γ -tubulin aggregates, depletion of the γ -TuRC component Dgp71WD or the Augmin protein Dgt5 caused little impact in the formation and function of cytoplasmic aMTOCs, suggesting that the mechanisms regulating MT nucleation from aMTOCs and those involving spindle-associated γ -tubulin are different.

Role of aMTOCs in Microtubule Cytoskeleton Remodeling during Mitotic Spindle Disassembly

Similar to what we observe during spindle assembly, several γ -tubulin aggregates dispersed in the cytoplasm were found to nucleate MTs de novo as mitotic spindles disassemble and cells transit into G1 stage (Figure 8). Surprisingly, very little is known about this process because most of the attention has been focused in understanding how spindle MTs are organized. We found that CDK1 down-regulation triggers cytoplasmic MT nucleation after anaphase, supporting that MT cytoskeleton remodeling at the exit from mitosis in animal somatic cells does not exclusively rely on centrosomes and is a cell cycle-regulated process. The mitosis–G1 transition has been shown to be accompanied by a redistribution of tubulin at an essentially constant polymer level (Zhai and Borisy, 1994), which means that, as kinetochore microtubules disassemble during anaphase, new microtubule polymer has to be formed. This is likely to be due to the increased microtubule stability and growth after anaphase onset but is also consistent with de novo microtubule nucleation at this stage. Our data further indicates that microtubule nucleation activity from cytoplasmic aMTOCs is promoted after anaphase onset, strongly suggesting that this process is under the control of the APC. The APC is responsible for the degradation of cyclin B at the metaphase–anaphase transition, thereby negatively regulating the activity of CDK1 and consequently microtubule dynamics (Verde *et al.*, 1990; Wheatley *et al.*, 1997). In agreement, assembly of equatorial MTOCs at the end of anaphase in fission yeast was also shown to require the activity of the APC (Heitz *et al.*, 2001).

Our results further implicate Cnn in the normal architecture and function of aMTOCs, which affects MT stability upon nucleation from noncentrosomal sites after CDK1 down-regulation. The first conclusion from these data are that MT nucleation events can occur from cytoplasmic sites that lack aMTOCs. However, we propose that these are important to sustain MT growth and confer stability to noncentrosomal nucleated MTs. In agreement with our results, de novo MT nucleation at nonspindle pole body MTOCs during mitotic exit in fission yeast was shown to require the Cnn-related protein mod20p, which binds and recruits γ -tubulin to these sites (Sawin and Tran, 2006). In *Drosophila*, Cnn has been shown to recruit γ -tubulin to centrosomes but also to form “flares” (Megraw *et al.*, 2002) that look similar to the γ -tubulin-containing aMTOCs described in this work. However, despite the coexistence of Cnn flares with centrosomes, they appear to lack γ -tubulin (Megraw *et al.*, 2002). In our hands, Cnn colocalizes with many endogenous γ -tubulin aggregates throughout mitosis and has a role in their formation and function. These results may be reconciled by the fact that detection of cytoplasmic γ -tubulin aggregates in the presence of centrosomes by fluorescence microscopy is difficult and requires oversaturation of the γ -tubulin signal at centrosomes, as well as highly sensitive detection devices.

We further found that Msps/XMAP215 and KLP10A critically affect net MT growth from aMTOCs upon down-regulation of CDK1. Members of the XMAP215 protein family were shown to be phosphorylated by CDK1 in vitro and in vivo and this modification decreases the MT elongation rate and stability (Vasquez *et al.*, 1999; Aoki *et al.*, 2006). Down-regulation of CDK1 during anaphase might alter the balance with protein phosphatases that control the phosphorylation state of XMAP215 (Tournebise *et al.*, 1997) and promote net MT growth from aMTOCs. This must be coordinated with the regulation of proteins that promote MT ca-

tastrophes, such as KLP10A, in order to maintain proper MT dynamics as cells exit mitosis. Interestingly, Msps/XMAP215 was recently implicated in acentrosomal MT formation from multiple cytoplasmic sites during interphase in *Drosophila* S2 cells (Rogers *et al.*, 2008). Importantly, the effect of depleting Msps/XMAP215 in MT regrowth after depolymerization was similar but not additive to that observed after γ -tubulin depletion, suggesting that these proteins work in the same pathway. Finally, CLASPs, which have been recently implicated in MT nucleation from the Golgi apparatus (Efimov *et al.*, 2007), appear to have no role in MT formation from aMTOCs in S2 cells, suggesting the existence of multiple pathways involved in noncentrosomal MT organization in animals.

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