A Multi-Component Assembly Pathway Contributes to the Formation of Acentrosomal Microtubule Arrays in Interphase Drosophila cells

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Running Head:
Identification of MT-Assembly Factors

Abbreviations:
MT, microtubules; HTM, high-throughput microscopy; MTOC, microtubule-organizing center; PCM, pericentriolar material; NEB, nuclear envelope breakdown; γTub, gamma tubulin; +TIP, plus-end tracking protein; Msps, mini-spindles; DHC, dynein heavy chain; RNAi, RNA interference

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ABSTRACT

In animal cells, centrosomes nucleate microtubules that form polarized arrays to organize the cytoplasm. Drosophila presents an interesting paradox however, as centrome-deficient mutant animals develop into viable adults. To understand this discrepancy, we analyzed behaviors of centrosomes and microtubules in Drosophila cells, in culture and in vivo, using a combination of live-cell imaging, electron microscopy, and RNAi. The canonical model of the cycle of centrosome function in animal cells states that centrosomes act as microtubule-organizing centers throughout the cell cycle. Unexpectedly, we found that many Drosophila cell-types display an altered cycle, in which functional centrosomes are only present during cell division. Upon mitotic exit, centrosomes disassemble producing interphase cells containing centrioles that lack microtubule-nucleating activity. Furthermore, steady-state interphase microtubule levels are not changed by co-depleting both γ-tubulins. However, γ-tubulin RNAi delays microtubule re-growth after depolymerization, suggesting that it may function partially redundantly with another pathway. Therefore, we examined additional microtubule nucleating factors and found that Mini-spindles, CLIP-190, EB1, or dynein RNAi also delayed microtubule re-growth; surprisingly, this was not further prolonged when we co-depleted γ-tubulins. Taken together, these results modify our view of the cycle of centrosome function and reveal a multi-component acentrosomal microtubule assembly pathway to establish interphase microtubule arrays in Drosophila.
INTRODUCTION

The microtubule (MT) cytoskeleton is essential for organizing the cytoplasm, polarity establishment, cell motility, morphogenesis, and cell division. Polarized arrays of MTs are nucleated by the centrosome, an organelle consisting of a pair of ‘mother-daughter’ centrioles that recruit and organize a surrounding matrix of pericentriolar material (PCM) (Bornens, 2002). Proteomic analyses of centrosomes identified several hundred different proteins, some of which are unique to centrioles (e.g., the structural subunits SAS-4, SAS-6 and SPD-2) or the PCM (e.g. pericentrin) (Andersen et al., 2003; Leidel and Gonczy, 2005a; Keller et al., 2005). Within the PCM, pericentrin and other coiled-coil proteins assemble into a scaffold that docks γ-tubulin ring complexes, which nucleate MT growth (Moritz and Agard, 2001). By manipulating centrosome number and position, cells exert precise control over the MT arrays needed for a variety of critical MT-dependent processes.

Centrosomes adhere to a cycle of duplication and function that is intimately coupled to the cell cycle (Tsou and Stearns, 2006). Centrosomes act as dominant MT-organizing and nucleating centers (MTOCs) (Schiebel, 2000), however, this activity is modulated in a cell cycle-dependent manner. During interphase, cells possess two paired centrioles that form a single centrosome and organize the interphase MT array. Prior to mitotic entry the centrosome duplicates once; the two centrosomes then separate from one another and undergo ‘maturation’, recruiting more PCM to nucleate additional MT growth and facilitate spindle assembly (Glover, 2005). Upon mitotic exit, each daughter cell receives a single centrosome that reduces its amount of associated PCM (Dictenberg et al., 1998), but remains active as an MTOC. γ-tubulin is thought to be the primary source of microtubule-nucleating activity from the centrosome (Wiese and Zheng, 2006), although it is likely not the only protein capable of this task. For example, developing Drosophila ovaries that harbor homozygous double mutations in both γ-tubulin genes (γTub23C and γTub37C) organize abnormal spindles within mitotic germ cells but are still competent to nucleate MT growth (Tavosanis and Gonzalez, 2003). Likewise, RNA interference (RNAi) of the single γ-tubulin gene in C. elegans leads to defects in spindle bipolarity but does not
abolish MT nucleation (Strome et al., 2001; Hannak et al., 2002). These studies suggest that alternative 
\(\gamma\)-tubulin-independent MT-assembly pathways exist in cells.

The importance and functional versatility of centrosomes is illustrated by their capacity to build 
several different MT-based machines including cilia, flagella and mitotic spindles (Rieder et al., 2001). 
This suggests that centrosomes should be essential for cell function. A recent study tested this 
hypothesis using *Drosophila* sas-4 mutants that fail to form centrioles and, therefore, centrosomes 
(Basto et al., 2006). Surprisingly, zygotic mutant embryos developed into viable adults with near normal 
timing and morphology. Although centrosomes usually organize mitotic spindle poles and were thought 
to be important for high fidelity chromosome transmission, dividing sas-4 mutant cells displayed few 
errors in chromosome segregation, because their chromosomes induced spindle assembly via an 
acentrosomal pathway (Basto et al., 2006). Similarly, mutant flies that lack Centrosomin, a PCM 
component essential for centrosome function, undergo normal zygotic development to form viable 
adults (Megraw et al., 2001). Importantly, these studies raised new questions: how do interphase 
*Drosophila* cells nucleate MT growth, organize their cytoplasm, and survive without the polarized MT 
arrays that centrosomes provide?

Much of our understanding of the cycle of centrosome function in *Drosophila* is derived from work 
in early embryos and asymmetrically dividing larval neuroblasts. During the rapid mitotic divisions of 
the early syncytial embryo, gap phases of the cell cycle (G1 and G2) are absent. Nonetheless, 
centrosome duplication and function largely follows the canonical cycle observed in most animal cells, 
as the centrosomes act as MTOCs throughout this cell cycle (Callaini and Riparbelli, 1990). Larval 
neuroblasts, however, display a novel twist to the classic cycle that is used to more precisely position the 
mitotic spindle (Rusan and Peifer, 2007; Rebollo et al., 2007). Following asymmetric division in these 
stem cells, the centriole pair separates as expected, but only one centriole (the “dominant centriole”) 
retains its PCM and MTOC activity during the intervening interphase, while the other sheds its PCM 
and is inactive with respect to MT nucleation. Prior to entering the next division, the inactive centriole
moves to the opposite side of the neuroblast, matures into a functional centrosome and is eventually segregated into the smaller ganglion mother cell. Although these two specialized cell-types possess functional centrosomes throughout their respective cell cycles, it is not clear how other Drosophila cells govern their centrosome cycles.

Here, we examine the Drosophila cycle of centrosome function both in cultured cells and within developing animals. Contrary to models of the conventional cycle, we find that Drosophila cells typically utilize centrosomes as MTOCs exclusively during mitosis. As cells exit mitosis, centrosomes disassemble both in cycling cells and within interphase-arrested cells. Furthermore, the generation and arrangement of interphase MTs appears to be independent of centrioles, and is not disrupted by γ-tubulin RNAi at steady-state. However, interphase MT re-growth assays reveal a ‘fast’ MT-assembly pathway that employs not only γ-tubulin but Mini-spindles, CLIP-190, EB1, and dynein. Our results suggest that Drosophila cells utilize a distinctive “canonical” cycle of centrosome function, in which interphase centrosomes are inactivated, being replaced by an alternative mechanism of organizing the interphase MT array. This provides a mechanistic explanation for the survival of centrosome-deficient flies.
MATERIALS AND METHODS

Cell Culture and Double-stranded RNAi

*Drosophila* cell culture and RNAi were performed as described (Rogers et al., 2002) with the following modifications. Cells were cultured in Sf900II SFM media (Invitrogen) without FBS. RNAi was conducted in 6 well plates and cells (50-90% confluency) were treated with 10μg of dsRNA in 1ml of media and replenished with fresh media/dsRNA every day for 7 days. ML-DmD16-c3 cells were obtained from the DGRC. Gene specific primer sequences are shown in Supplementary Table 1. The efficiency of RNAi was determined by Western blotting S2 lysates where overall protein amounts loaded were normalized and verified using antibodies against α-tubulin. Percent depletion of the target protein was measured using the densitometry functions of ImageJ.

Immunofluorescence Microscopy

For immunostaining, S2 cells were fixed exactly as described (Rogers et al., 2002) with either -20°C methanol or 10% formaldehyde. Antibodies used in this study were diluted to concentrations ranging from 1-20 μg/ml (anti-SAS-6 [our lab], D-PLP [J. Raff, University of Cambridge], DM1a α-tubulin [Sigma], Cnn [R. Jones, UT Southwestern], CP60 and CP190 [M. Moritz, UCSF], E7 α-tubulin [Developmental Studies Hybridoma Bank], GTU-88 [Sigma], phosphohistone H3 [Upstate, Cell Signaling], GFP [LabVision #MS-1315-PO], anti-Golgi membrane protein [Calbiochem #345867]). Secondary antibodies (Cy2 and Rhodamine Red [Jackson ImmunoResearch]) were used at final dilutions of 1:100. Cells were mounted in a 0.1M propyl galate-glycerol solution. DAPI (Sigma) was used at a concentration of 5μg/ml. For cold-treatments, S2 cells pre-spread on concanavalin A (conA) dishes were placed on a partially submerged aluminum block in an ethanol-ice bath, placed at 4°C for 1 hour, and allowed to recover at either 38°C or room temperature depending on the ambient temperature. For fixed embryo analysis, WT embryos were dechorionated with 50% household bleach and then placed in a 1:1 3.7% formaldehyde in PBS:heptane for 20 min. Embryos were devitellinized in methanol and then blocked and stained in PBS/1% goat serum/0.1% Triton X-100. Specimens were imaged using
either a TE2000-E Nikon microscope or a Yokogawa spinning-disk confocal (Perkin Elmer) mounted on a TE300 Nikon microscope.

**Antibodies**

*E. coli*-expressed GST- or MPB-SAS-6 (full-length) and D-PLP (amino acids 8-351) proteins were purified on either glutathione-Sepharose or amylose resin (Martinez-Campos *et al.*, 2004). Rabbit polyclonal antisera (ProteinTech) were raised against affinity-purified fusion proteins that were also precoupled to Affigel 10/15 (Bio-Rad) and used for antibody affinity purification performed by elution with low pH buffer.

**Live Cell Microscopy**

Endogenous promoters for GFP constructs were made by PCR of genomic regions upstream of γTub23C (761bp), γTub37C (476bp) or SAS-6 (208bp) and cloned into pMT vectors (Invitrogen). Stable cell lines were generated using Effectene transfection reagent (Qiagen)/pCoHygro selection system (Invitrogen) and plated on 0.5mg/ml conA-coated glass-bottom dishes (MatTek) for 1hr prior to observation. Cells were imaged with a 100X 1.45 NA PLAN APO objective using a TE2000-E Nikon microscope equipped with a Cascade 512B cooled CCD camera (Roper) or a Yokogawa spinning disk confocal (Perkin Elmer) mounted on a TE300 Nikon microscope equipped with an ORCA-ER cooled CCD camera (Hamamatsu). For *in vivo* live imaging, embryos of the genotype Gal4(nos-NGT40)/mCherry::SAS6;+/UAS-EB1::GFP were dechorionated with 50% house-hold bleach, covered in halocarbon oil (series 700; Halocarbon Products Corporation) and mounted between a #1.5 glass coverslip and a gas-permeable membrane (petriPERM; Sigma). Embryos were then imaged using the spinning-disk microscope mentioned earlier. Time-lapse sequences were collected using MetaMorph (Molecular Devices). All stable cell lines expressing fluorescent proteins will be made available through the DGRC.

**Transmission Electron Microscopy**
Cell monolayers grown on polystyrene plates were rinsed with PBS and fixed in 3% glutaraldehyde with 0.1M sodium cacodylate, pH 7.4, for several hours or overnight. After buffer washes, the monolayers were postfixed for 1 hour with 1% osmium tetroxide, 1.25% potassium ferrocyanide, 0.1M sodium cacodylate buffer. The cells were dehydrated using increasing concentrations of ethanol, infiltrated and embedded in Polybed 812 epoxy resin (Polysciences, Inc.). The blocks were sectioned parallel to the substrate at 70nm using a diamond knife, and the sections were mounted on 200 mesh copper grids followed by staining with 4% aqueous uranyl acetate and Reynolds’ lead citrate. Sections were observed with a LEO EM910 transmission electron microscope operating at 80kV (LEO Electron Microscopy) and photographed using a Gatan Orius SC1000 CCD Digital Camera and Digital Micrograph 3.11.0 (Gatan, Inc.).

**High-Throughput Microscopy**

S2 cells were seeded in conA-coated 24-well glass-bottom plates (MatTek) for 1hr prior to fixation, stained (described above) and scanned with either an IC100 Image Cytometer (Beckman Coulter) or an Array Scan VTI (Cellomics) equipped with a 20X 0.5 NA or 40X 0.95 NA objective and an ORCA-ER cooled CCD camera. Images of ~5,000 cells per well were acquired and analyzed using CytoShop v2.1 (Beckman-Coulter) or vHCS View (Cellomics). Integrated fluorescence intensity measurements were determined from unsaturated images.
RESULTS

Multiple Drosophila Cell Types Lack Functional Centrosomes or MT-Organizing Centers (MTOCs) During Interphase

The canonical model for centrosome function in most animal cells suggests that interphase MTs are nucleated at the centrosome, to provide a polarized array that organizes the cytoplasm (Schiebel, 2000). However, in apparent contradiction to this vital centrosomal function, zygotic centrosome-deficient mutant fly embryos develop into viable adults (Megraw et al., 2001; Basto et al., 2006). We investigated this discrepancy by examining the cycle of centrosome function in Drosophila cells.

The ability to use RNAi to generate loss-of-function phenotypes in cultured S2 cells makes them a powerful system for studying cytoskeletal cell biology (Rogers et al., 2004a). We initially used antibodies against γ-tubulin to visualize centrosomes, as it is a conserved PCM component and a widely used centrosome-marker. As expected, MTOCs appear as hollow spheres of γ-tubulin at the center of MT asters during early prophase, metaphase and throughout cytokinesis (Figure 1 A-C).

Surprisingly, anti-γ-tubulin antibodies failed to recognize a discrete MTOC in interphase Drosophila S2 cells. Instead, γ-tubulin immunolocalized in a diffuse pattern throughout the cytoplasm as small punctae (Figure 1 D); these were abolished by γ-tubulin RNAi (Supplemental Figure 1). Consistent with the absence of an MTOC, MTs appeared to be broadly distributed and non-radial, in contrast to the polarized radial arrays in cultured interphase vertebrate cells (Wiese and Zheng, 2006).

As an independent method to examine γ-tubulin distribution, we engineered a stable S2 line expressing γ-tubulin-GFP under control of the gene's endogenous promoter. Stable cell lines expressing γ-tubulin-GFP had mitotic MTOCs equal in number to those observed in wild-type S2 cells stained with anti-γ-tubulin (data not shown). Consistent with the immunostaining, γ-tubulin-GFP concentrated into hollow spheres at spindle poles and was uniformly diffuse throughout the cytoplasm in interphase (Figure 1E and F).
We next examined whether this centrosome behavior was unique to S2 cells. We found that cultured *Drosophila* D16 cells, an imaginal disc-derived cell line (Ui et al., 1987), also contained γ-tubulin-MTOCs during mitosis but lacked these structures during interphase (Supplemental Figure 2). To examine whether this was also true *in vivo*, we examined three embryonic cell types during the process of dorsal closure (Figure 2). MT staining revealed that amnioserosal and leading edge cells, two differentiated cell-types terminally arrested in interphase, displayed MT arrays lacking a central focus. Similar to cultured cells, MTs in amnioserosal cells were broadly distributed (Figure 2A; panel 1), whereas MT were arranged into elongated bundles that spanned the long-axis of leading edge cells, as previously described (Figure 2A; panel 2) (Jankovics and Brunner, 2006). Both cell types lacked a concentrated punctate γ-tubulin staining that would indicate the presence of MTOCs (Figure 2B; panels 1 and 2). In contrast, typical γ-tubulin-labeled centrosomes were observed at mitotic spindle poles within dividing cells of the embryo (Figure 2; panel 3 and inset).

In the canonical animal cell cycle, centrioles recruit PCM and are active MTOCs throughout the cell cycle. Our observations above suggest either that interphase *Drosophila* centrioles are absent, which we considered unlikely, or that they fail to recruit PCM and act as MTOCs. To test these hypotheses, we developed tools to examine centrioles in living and fixed cells. We generated a stable S2 line expressing GFP (or mCherry) fused to the fly SAS-6 protein under control of the gene’s endogenous promoter. SAS-6 is a conserved structural component of centrioles (Dammermann et al., 2004; Leidel et al., 2005b) and *Drosophila* sas-6 mutants lack basal bodies at sensory bristles and centrioles within larval brains (Peel et al., 2007). We confirmed that our GFP-SAS-6 construct is a reliable marker for centrioles as it co-localized with D-PLP, a known centriole component (Martinez-Campos et al., 2004) (Supplemental Figure 3A). Furthermore, affinity-purified anti-SAS-6 antibodies (generated against full-length recombinant fly SAS-6) labeled spots within γ-tubulin spheres in mitotic cells, as we saw with GFP-SAS-6 (Supplemental Figure 5A). Stable expression of GFP-SAS-6 did not increase interphase centriole number (data not shown).
As expected, GFP-SAS-6-marked centrioles recruit PCM in mitotic cells (Figure 3A). During interphase, however, centrioles did not recruit γ-tubulin, as GFP-SAS-6 appeared as discrete spots in the cytoplasm that did not obviously co-localize with γ-tubulin punctae (Figure 3B). To test if this failure to be recruited was unique to γ-tubulin, we examined the distributions of other PCM components including Centrosomin (Cnn), CP190, and CP60 (Oegema et al., 1995; Butcher et al., 2004; Li and Kaufman, 1996). As with γ-tubulin, each protein localized to centrosomes at mitotic spindle poles, but not with centrioles in interphase S2 cells (Supplemental Figure 4). We next assessed whether this failure to recruit PCM during interphase was unique to S2 cells. Instead, failure to recruit PCM during interphase appears to be the standard behavior of Drosophila cells: we observed γ-tubulin recruitment to PLP-labeled centrioles in mitotic but not interphase D16 cells (Supplemental Figure 3B). This behavior was also observed in vivo during normal development: centrioles recruited γ-tubulin in mitotic embryonic cells and in dividing embryonic neuroblasts (Figures 3D, F; yellow arrowheads mark centriole and γ-tubulin positions, and blue actin-labeling denotes the borders of adjacent cells) while centrioles within non-dividing leading edge and amnioserosal cells lacked γ-tubulin staining (Figures 3C, E; yellow arrowheads). In some cycling cells, both in culture and in vivo, small amounts of γ-tubulin staining could be detected on interphase centrioles (data not shown). We hypothesize that this may be due to a gradual recruitment or loss of PCM as centrioles prepare to enter or exit mitosis.

The co-localization of γ-tubulin on mitotic but not interphase centrioles suggested that functional centrosomes are not present in interphase cytoplasm and therefore centrioles would not associate with MTs in a manner characteristic of MTOCs. To determine this relationship in fixed S2 cells, we examined centrioles at higher resolution by electron microscopy. Centrioles were clearly identified as electron-dense structures approximately 0.2μm in length and exhibiting a ring of nine doublet MT-bundles, a typical arrangement in Drosophila (Figures 3G' and I') (Gonzalez et al., 1998). Centrioles in mitotic cells appeared as doublets where a mature “mother” centriole was in close proximity to smaller “daughter” centriole of variable size (Figure 3G), consistent with their duplication in the previous S-
phase. We noticed that in some interphase cells, centrioles appeared as singlets that had not yet duplicated (compare Figures H' and I'). As expected for functional centrosomes, centriole doublets in mitotic cells were surrounded by a dense network of MTs (prophase cell shown in Figure 3G). In contrast, centrioles in interphase cells did not display this feature and did not appear tightly associated with MTs, although nearby MTs were easily identified (Figures 3H and I). Thus, based on our analysis of fixed cells, *Drosophila* displays a unique cycle of centrosome function that directs the inactivation of interphase centrosomes and MTOCs.

**Loss of Centrioles Does Not Cause G1 Arrest or Delay**

Previous studies demonstrated a critical role for centrosomes in G1 progression through the cell cycle in cultured mammalian cells (Hinchcliffe *et al.*, 2001; Khodjakov and Rieder, 2001; Mikule *et al.*, 2007). However, other studies suggested that centrosomes are not required (Uetake *et al.*, 2007). In *Drosophila*, centriole-deficient larvae develop to adults with near normal timing (Basto *et al.*, 2006) suggesting that, in this system, centrioles do not influence cell cycle progression, but an analysis of the cell cycle had not been performed. Therefore, to test whether fly centrioles regulate cell cycle progression, we depleted S2 cells of SAS-6 using RNAi and examined their cell cycle distributions. Anti-SAS-6 Western blots confirmed that RNAi treatment depleted protein levels by >99% (Supplemental Figure 5B) and effectively eliminated D-PLP stained centrioles (Supplemental Figure 5C). Whereas control-treated interphase cells contained a median centriole number of 2.0 centrioles (avg. 3.0 ± 3.2; 654 cells counted), SAS-6 depleted cells contained a median centriole number of 0 (avg. 0.2 ± 0.6; 425 cells counted) (Supplemental Figure 5D). Furthermore, SAS-6 depletion slightly increased the mitotic index (3.4% vs. 2.5% in control) and dramatically elevated the frequency of mitotic bipolar spindles with no centrosomes (Supplemental Figure 5E). These phenotypes are very similar to those described for centriole-deficient larval neuroblasts (Martinez-Campos *et al.*, 2004), and are consistent with a role for *Drosophila* SAS-6 in centriole assembly. If *Drosophila* SAS-6 and centrioles played an important role in progression through G1, we would expect that loss of SAS-6
would dramatically alter the cell cycle distribution and decrease the number of cells within the G2-phase peak. However, quantitative high-throughput microscopy (HTM) revealed that control and SAS-6 RNAi treated cells had indistinguishable cell cycle profiles (Supplemental Figure 5F). Therefore, centrioles are likely not essential for interphase cell cycle progression in *Drosophila* cells. Similar results were observed in S2 cells depleted of Plk4, a kinase required for centriole duplication (Bettencourt-Dias *et al.*, 2005), as well as after SAS-6 siRNA in human cells (Strnad *et al.*, 2007). However, unlike human SAS-6, which is degraded from anaphase until late G1 phase (Strnad *et al.*, 2007), fly SAS-6 is a stable centriole marker throughout the cell cycle (our unpublished results).

**Interphase Microtubule Arrays are Nucleated Independently of Centrioles**

These data suggest that in many *Drosophila* cell types centrosomes are inactivated in interphase. In the canonical animal cycle of centrosome function, centrosomes serve as interphase MTOCs. To assess relationships between centrioles and MT dynamics, we generated a stable S2 line co-expressing GFP-SAS-6 and EB1-mRFP as well as transgenic flies co-expressing mCherry-SAS-6 and EB1-GFP. EB1 is a MT plus-end tracking protein (+TIP) and can be used to visualize growing MT plus-ends (Rogers *et al.*, 2002). We recorded living interphase and mitotic cells by time-lapse microscopy and analyzed the patterns of MT growth relative to centrioles. In S2 cells, EB1-labeled MTs were nucleated at many discrete sites in the cytoplasm of interphase cells and did not emanate from centrioles, which exhibited random movements near the cell periphery (Figure 4A; see Movie 01). In contrast, upon entry into mitosis MT nucleation originated from centrioles (Figure 4B; see Movie 02). We also examined MT dynamics in several embryonic cell types. In live amnioserosal and leading edge cells (arrested in G2 and G1 phase, respectively), centriole position oscillated throughout the cell and MT nucleation occurred at sites unrelated to centriole location (Figure 4C and D; see Movies 03 and 04). Interestingly, in leading edge cells MT organization is not random, but instead is oriented along the long axis of the cell (Figure 4D) (Jankovics and Brunner, 2006), but centrosomes do not appear to play a role in this organization. In contrast, EB1-tracks emanated from centrioles at spindle poles within dividing
embryonic ectodermal cells (Figure 4E; Movie 05). Thus, in each *Drosophila* cell type examined, centrioles possess MTOC activity only during mitosis and do not appear to influence interphase MTs. However, as EB1 only labels growing MT plus-ends, we cannot rule out the possibility of rapid nucleation and release of some MTs at centrioles.

**Steady-State Interphase Microtubule Levels are Insensitive to γ-Tubulin Depletion**

We next investigated the molecular requirements for establishing acentrosomal interphase MT arrays in S2 cells by using RNAi against proteins with known MT-nucleating activity. To begin, we focused on γ-tubulin, the central MT-nucleation factor in eukaryotic cells (Wiese and Zheng, 2006). The *Drosophila* genome encodes two γ-tubulin genes: γTub23C, the major isotype in S2 cells, and γTub37C, the minor isotype (Raynaud-Messina et al., 2004). In a previous study, RNAi of γTub23C alone did not seem to affect the interphase MT cytoskeleton (Raynaud-Messina et al., 2004). However, these authors were not able to deplete the minor γTub37C and it remained possible that γTub23C and γTub37C function redundantly to nucleate the interphase MTs. Indeed, functional redundancy has been reported for the two γ-tubulins during female germ-cell development and oogenesis (Tavosanis and Gonzalez, 2003). Recently, we discovered that RNAi is much more effective if S2 cells are cultured in the absence of fetal bovine serum and have modified our RNAi procedure (data not shown) (see Materials and Methods). Using double-stranded RNA (dsRNA) that targets the 3'UTR, γTub23C RNAi was highly effective (below the level of detection) as determined by Western blotting (Figure 5B). Depletion of γTub23C produced the expected mitotic phenotype, elevating the mitotic index by 4-fold and produced numerous monopolar spindles (Figure 5C), as previously described (Raynaud-Messina et al., 2004), suggesting we reduced its levels below a critical threshold. However, interphase cells in the same population still contained MTs, and MT nucleation and growth, as assessed by live cell imaging of EB1-mRFP and GFP-SAS-6, was indistinguishable from wild-type cells (Figure 5F; see Movie 06). Rates of fluorescent EB1 movement revealed that MT growth was similar in control and γTub23C depleted cells (control 9.9 ± 4.0μm/sec [30 MTs in 3 cells]; γTub23C 7.9 ± 2.0μm/sec [30 MTs in 4 cells]).
Due to the low abundance of γTub37C, our γ-tubulin antibody did not detect this minor isotype in Western blots of S2 cell lysates. In order to determine the efficacy of RNAi, we monitored depletion by generating a γTub37C-GFP stable S2 line driven by the gene’s endogenous promoter. This approach allowed adequate expression to detect by Western blotting using antibodies against γ-tubulin (Figure 5B) or GFP (data not shown). Like γTub23C, γTub37C-GFP localized to hollow spheres at mitotic spindle poles but was diffuse in the cytoplasm of interphase cells and did not co-localize with centrioles (Figure 5A). After RNAi treatment using a 1.1kb dsRNA that targets a large segment of the γTub37C ORF, γTub37C-GFP levels were reduced below the level of detection (Figure 5B). Due to the high nucleotide identity (78%) between the two γ-tubulins within this coding region, γTub23C was also efficiently depleted by γTub37C RNAi (Figure 5B). Similar “cross-depletion” of γTub23C was observed in a previous study (Raynaud-Messina et al., 2004). As expected, this treatment elevated the frequency of monopolar spindle formation (Figure 5C), as did the co-depletion of γTub23C/37C where dsRNAs against both γTub23C/37C were added to the culture (Figure 5B).

We found that interphase cells depleted of γTub23C or γTub37C individually or together assembled MTs and appeared similar to control-treated cells (Figure 5D). To quantitate changes in MT levels, S2 cells were fixed in glass-bottom 24-well plates, stained for α-tubulin and scanned using quantitative HTM. Single γ-tubulin “knockdowns” or γTub23C/37C co-depleted cells revealed no significant difference in the mass of MT polymer compared to control-treated cells (Figure 5E). Thus, our observations of acentrosomal steady-state MT-assembly suggest the presence of a γ-tubulin-independent or redundant mechanism for generating interphase MT arrays.

**MT Re-growth Does Not Occur From Interphase Centrioles**

To examine the possible sites of MT nucleation with more clarity, and to examine the role of γ-tubulin in this process, we cold-treated S2 cells to induce MT depolymerization and analyzed the position of centrioles relative to the sites of re-growth of MTs, by fixing the cells at 0, 2.5, 5, 10, 15 and
30 min time-points. When chilled for 1 hour, MTs completely depolymerized in both mitotic and interphase S2 cells (Figure 6A, 0 min; data not shown). Within 5 minutes after a return to room temperature, robust MT re-growth occurred from centrioles in dividing cells (Figure 6A, 5min cytokinesis). In contrast, MT re-growth in interphase cells did not occur from centrioles; small MTs were first observed by 2.5 min (Supplemental Figure 6A, 2.5 min). By 5 min, MTs appeared at many sites within the cytoplasm, as individual MTs or as small tufts of MTs (Figure 6A). In some cells, some MT foci associated with centrioles but most did not (Supplementary Figure 6A, 5 min). At 10 min, MT foci were more numerous and assembled into an extensive interconnecting MT network throughout the cell (Figure 6A, 10 min). By 15 min, S2 cells displayed a normal interphase array, having dispersed the MT foci that formed in the earlier time-points (Figure 6A, 15 min).

Previous MT re-growth experiments using cultured fly cells described the appearance of unique MT foci similar to what we observed (Colombie et al., 2006; Cottam et al., 2006). Although individual MTs and foci form independently of interphase centrioles in S2 cells (our findings, Figure 6A), γ-tubulin co-localizes with MT foci that assemble in cold-treated Clone8+ cells (a wing imaginal disc-derived cell line) (Cottam et al., 2006). In contrast, we found that in cold-treated S2 cells γ-tubulin did not co-localize with MT foci, although γ-tubulin punctae were apparent in interphase (Figure 6B; bottom panel), and γ-tubulin strongly co-localized with centrioles and active MTOCs in mitotic cells (Figure 6B; upper panel).

Next, we examined whether the Golgi apparatus acts as the assembly site of MT foci in cold-treated S2 cells, as non-centrosomal MT nucleation from the Golgi apparatus is described in mammalian cells (Chabin-Brion et al., 2001; Efimov et al., 2007). Golgi in interphase S2 cells appear as numerous discrete punctae widely distributed throughout the cytoplasm (Stanley et al., 1997). Using a monoclonal antibody that recognizes an integral membrane protein enriched in Golgi membrane fractions, we found that Golgi were restricted to the center of S2 cells that were spread on concanavalin A-coated glass. Five min after recovery from cold-treatment, MTs were apparent in the thin extended lamella, a region
devoid of Golgi (Figure 6C, 1), and some MT foci in the more central region were not associated with Golgi punctae (Figure 6C, 2) suggesting that not all foci nucleate from Golgi. However, Golgi were visible at the center of some MT foci (Figure 6C, 3); this was also observed at an earlier time-point (Supplemental Figure 6B; 2.5 min). Golgi also appeared as linear tracks of punctae associated with long MT bundles (Figure 6C, 4). The appearance of most MTs at sites not coincident with Golgi suggests that Golgi is not essential for non-centrosomal MT nucleation in S2 cells. However, we cannot rule out the possibility that Drosophila Golgi has MT-nucleating capability, as some MT association is observed.

**MT Re-growth Is Delayed Without γ-Tubulin**

We next used our assay to directly examine whether γ-tubulin plays a role in the kinetics of MT re-growth and the formation of MT foci. Double γ-Tub-23C/37C RNAi did not prevent the assembly of MT foci (Figure 6D). However, γ-tubulin depletion altered the rate of MT recovery in interphase cells (Figure 6D and E). By 15 min, 94% of control RNAi-treated cells fully recovered their MT arrays, whereas only 3% of γ-tubulin depleted cells fully recovered at this time. In the absence of γ-tubulin, cells required an additional 15 min to complete full recovery (Figure 6E). We conclude that γ-tubulin is likely not required for maintaining MT levels at steady-state in interphase S2 cells but is required for normal kinetics of MT re-growth after cold treatment. Our findings also suggest that cold-recovering interphase cells utilize a biphasic pathway to assemble acentrosomal MT arrays, where γ-tubulin contributes to an initial fast phase of assembly.

**γ-Tubulin, Mini-spindles, CLIP-190, EB1, and Cytoplasmic Dynein Contribute To a Fast MT-Assembly Pathway During MT Re-growth**

Given our findings that γ-tubulin is dispensable for maintaining steady-state MT levels and that MTs eventually fully recover after cold-treatment, we explored other potential candidates that might participate in this putative γ-tubulin-independent or partially redundant mechanism. One potential candidate is CLASP; members of this conserved family of +TIPs regulate MT dynamic instability throughout the cell cycle. Recent work revealed that in human interphase cells, CLASP localizes to the
trans-Golgi network (TGN) where it can nucleate a non-centrosomal population of MTs (Efimov et al., 2007). MAST/Orbit is the only fly CLASP. Surprisingly, recent work revealed that while MAST promotes MT stability during interphase, and MAST RNAi reduces MT density as well as the number of MTs that extend to the cell cortex, MAST appears to play no role in MT nucleation in S2 cells, as the kinetics of MT re-growth after cold-depolymerization is normal (Sousa et al., 2007). In agreement with this, we found that MAST depletion dramatically elevated monopolar spindle formation (Figure 5C), and eliminated cortical MTs as expected (Supplemental Figure 7). Further, MAST depletion decreased steady-state MT levels (reducing the mean by approximately 34%) compared to controls (Figure 7A), validating our quantitative cytometric approach. However, MTs in MAST depleted S2 cells recovered from cold-treatment with normal kinetics, as previously described (Figure 7B; second row) (Sousa et al., 2007). These data left open the possibility that MAST and γ-tubulin play redundant roles in nucleating MTs. To test this hypothesis, we used RNAi to simultaneously deplete both proteins from S2 cells. At steady-state, these displayed MT levels slightly lower than those found in cells depleted of MAST alone (Figure 7A). Furthermore, MT re-growth in these cells recovered with the same kinetics observed after γ-tubulin RNAi alone; a 15 min delay in full recovery (Figure 7B; third row). Thus, simple redundancy between MAST and γ-tubulin seem unlikely.

We next tested another candidate, CLIP-190, the fly homolog of the CLIP-170 family of +TIPs (Dzhindzhev et al., 2005). They possess multiple conserved tubulin-binding CAP-Gly domains that can nucleate MT growth in vitro (Slep and Vale, 2007). Although not as dramatic as MAST RNAi, we found a significant change in steady-state MT levels in CLIP-190 depleted cells as compared to controls (Figure 7A). Unlike MAST, however, CLIP-190 RNAi altered the rate of MT re-growth after cold-induced depolymerization and delayed MT recovery by 15 min, similar to γ-tubulin depletion (Figure 7B, fourth row). If γ-tubulin and CLIP-190 function partially redundantly to nucleate MTs, then we expected that co-depletion of both proteins would produce a more severe MT assembly phenotype. However, MT steady-state levels were not further reduced by co-depletion as compared to CLIP-190
RNAi alone (Figure 7A) and, interestingly, cells recovered from cold-treatment with the same kinetics displayed in the single RNAi treatments (Figure 7B; fifth row). Thus, we conclude that CLIP-190 is not redundant with γ-tubulin in maintaining steady-state MT levels and thus may function in the same assembly pathway as γ-tubulin to nucleate a fast phase of MT re-growth.

A third candidate we examined is Mini-spindles (Msps), the sole fly member of the conserved Dis1/TOG family of MT-associated proteins (MAPs). Like MAST, these are +TIPs that promotes MT growth (van Breugel et al., 2003; Howard and Hymann, 2007). Msps RNAi inhibits MT plus-end growth and increases MT pausing and bundling in interphase S2 cells (Brittle and Ohkura, 2005). Members of this family contain an array of multiple conserved TOG domains that bind αβ tubulin heterodimers capable of inducing MT nucleation in vitro (Slep and Vale, 2007). We observed extensive cortical MT bundling after Msps depletion (Supplemental Figure 7), as well as a decrease in steady-state MT levels (43% reduction in mean polymer mass), but this did not diminish further upon co-depletion of γ-tubulin (Figure 7A). These data suggest that Msps and γ-tubulin are not redundant in maintaining MT levels at steady-state. However, as with γ-tubulin depletion, we found that Msps RNAi alone delayed the recovery of MTs after cold-treatment by ~15 min (Figure 7B; sixth row). To examine whether Msps could function in the eventual recovery of MTs that we observed after cold-treating γ-tubulin depleted cells, we performed Msps/γ-tubulin double RNAi. Surprisingly, these cells displayed a rate of MT re-growth similar to the single RNAi treatments; a delay of 15 min (Figure 7B; seventh row). Since we did not observe a synergistic effect by double Msps/γ-tubulin RNAi, our findings suggest that Msp may also function together with γ-tubulin in a fast MT-nucleation pathway.

Since our data implicate two +TIPs (CLIP-190 and Msps) in MT assembly, we next examined the contribution of EB1 in our functional assays, as EB1 recruits stabilizing +TIPs to growing MT plus-ends (Vaughan, 2005). Moreover, a recent in vitro study demonstrated that Mal3p, the S. pombe EB1 homolog, may induce polymer stability by binding along the MT lattice seam (Sandblad et al., 2006). Expectedly, EB1 depletion decreased overall steady-state MT levels, similar to our results with CLIP-
but not as dramatically as either MAST or Msps RNAi (Figure 8A). We also observed a modest
decrease in steady-state MT levels after EB1/γ-tubulin co-depletion (Figures 8A). Strikingly, we found
that EB1 RNAi alone delayed the kinetics of MT re-growth after cold-treatment by 15 min (Figure 8B;
second row). However, this delay was not further prolonged after co-depletion with γ-tubulin (Figure
8B; third row). Thus, these data suggest that EB1 may also function with γ-tubulin in an initial fast MT-
nucleation pathway.

We also examined whether the cytoplasmic dynein MT-based motor complex and its associated
adaptor complex, dyantin, participate in interphase MT assembly. Drug inhibition of dynein in fish
melanophore cell fragments greatly diminishes the rate of MT nucleation that is needed to build radial
acentrosomal arrays in this system, and dynein is effective in MT nucleation in vitro (Malikov et al.,
2004). Although dynactin does not nucleate MTs in vitro (Malikov et al., 2004), the p150Glued dynactin
component contains a conserved tubulin-binding CAP-Gly motif, also found in CLIP-190, suggesting a
possible role in MT nucleation in cells. Consistent with roles in MT stability, we found that either
dynein heavy chain (DHC) or p150Glued RNAi reduced steady-state MT levels (Figure 8A), although, as
with CLIP-190 RNAi, mean and median levels were not dramatically altered. Co-depleting γ-tubulin
did not further reduce MT levels (Figure 8A). MT re-growth assays revealed that DHC depletion alone
delayed the full recovery of the acentrosomal interphase array by approximately 15 min (Figure 8B;
fourth row), whereas p150Glued RNAi did not (Figure 8B; sixth row). These results suggest a dynactin-
independent function for DHC in Drosophila cells, consistent with the in vitro results of Malikov et al.
(2004). As before, we applied the MT re-growth assay to cells co-depleted of DHC or p150Glued with γ-
tubulin in order to determine their functional relationship in MT nucleation. Both p150Glued/γ-tubulin
RNAi and DHC/γ-tubulin displayed a 15 min delay in MT re-growth, similar to DHC or γ-tubulin RNAi
alone (Figure 8B; fifth and seventh rows). Thus, these results suggest that DHC, but not dynactin, also
contributes to a fast phase of MT nucleation during re-growth.
To determine whether these +TIPs and MT motors play specific roles in MT re-growth, we also examined several additional +TIP or MT-binding proteins for roles in interphase MT re-growth. These included the kinesins Ncd (a minus-end directed motor), conventional kinesin heavy chain (KHC), and the MT-depolymerase Klp10A, as well as the spectroplakin Shortstop (Shot). Ncd, Klp10A, and Shot all display +TIP behavior (Rogers et al., 2004b; Goshima et al., 2005; Mennella et al., 2005; Slep et al., 2005). Depletion of each of these proteins individually did not change rates of MT recovery as compared to control treatments (Supplemental Figure 8). Thus, +TIPs do not perform a general role in MT assembly. We note in passing that although the kinetics of MT recovery did not change after KHC RNAi, MT re-growth was restricted to perinuclear regions of the cell (Supplemental Figure 8).

Taken together, our findings suggest that interphase Drosophila cells utilize a biphasic pathway to assemble acentrosomal MT arrays, perhaps by γ-tubulin, CLIP-190, Msps, EB1 and DHC working together to drive an initial fast phase of MT assembly. Removal of any one component inactivates the fast phase and reveals the presence of a partially redundant, slower phase to ensure assembly of the MT array via an uncharacterized mechanism.

**DISCUSSION**

*The Typical Cycle of Centrosome Function in Drosophila Cells*

The current model for regulating MT nucleation in animal cells suggests that centrosomes play a key role in generating MT arrays in both mitosis and in interphase. Our analysis revealed that the Drosophila cycle of centrosome function is quite distinct from that of vertebrate cells, where centrosomes act as MTOCs throughout the cell cycle. In Drosophila cells, functional centrosomes are assembled at the onset of mitosis and participate in spindle assembly, but then disassemble upon mitotic exit. Interphase centrioles do not recruit γ-tubulin and, thus, lack the capacity to nucleate and organize MT arrays.

Our results provide mechanistic insight into the recent surprising report that centriole-deficient sas-4 mutant flies develop into viable adults with near normal timing and morphology (Basto et al., 2006). This study raised a fascinating question – how do interphase cells, in this mutant background,
survive without the cytoplasmic organization that centrosomes provide? Our results demonstrate that *Drosophila* cells do not depend on centrosomes to nucleate/organize interphase MTs or to progress through interphase, explaining why an absence of centrosomes has little impact on development.

Our study does not suggest that centrosomes are unimportant, however, only that they are not essential for interphase homeostasis, since they are normally absent during interphase in many cell-types. Centrosomes are present during mitosis however, and are vital for early divisions during cleavage, where they support proper spindle/nuclear spacing within the large syncytium. This was best shown for mutations in the PCM component, Centrosomin; its loss blocks centrosome function, producing spindle assembly defects and maternal-effect lethality (Megraw *et al.*, 1999). Centrosomes are also essential to assemble cilia and flagella; *sas-4* mutant adults lack cilia in their mechanosensory neurons, are severely uncoordinated, and die shortly after eclosion (Basto *et al.* 2006).

Roles for vertebrate centrosomes extend beyond MT organization. Several studies suggest that centrosomes provide an essential scaffold for docking numerous cell cycle regulators (reviewed in Doxsey *et al.*, 2005). This may explain why a cycle of centrosome function that utilizes an interphase centrosome was acquired or maintained in vertebrate evolution while centrioles are not essential for *Drosophila* cell cycle progression in culture. It would be interesting to manipulate the *Drosophila* centrosome to mimic the vertebrate cycle, in order to examine the impact of a persistent, functional centrosome on interphase MT-dependent processes at the cellular level as well as overall development of the animal.

**A New Mechanism of Nucleating Interphase Microtubules**

Our findings thus support a reassessment of the view that the centrosome is the major MT nucleating and organizing center in all interphase cells, producing a polarized radial MT array to organize the cytoplasm of interphase proliferating and migrating animal cells. Other evidence also supports the need for this reassessment. In some cell-types, such as epithelial cells and neurons, MTs are released from centrosomes and incorporated into non-centrosomal MT arrays (Waterman-Storer and Salmon, 1997;
Ahmad et al., 1999). Indeed, the MT nucleating capacity of the centrosome, and the fraction of MTs in
the cell that originate from the centrosome, can vary dramatically between cell types and species
(Waterman-Storer and Salmon, 1997). Finally, cycling vertebrate cells generally reduce the amount of
PCM associated with the centrosome when exiting mitosis (Dictenberg et al., 1998; Rusan and
Wadsworth, 2005). Thus, the cycle of centrosome activation/inactivation in Drosophila cells that we
describe here may represent an exaggerated version of behavior that is found in at least some vertebrate
cells.

If centrosomes do not function in interphase Drosophila cells, then what nucleates MT growth
and how is the cytoplasm organized without an MTOC? Using RNAi, we examined the roles of several
known and putative MT-nucleators in maintaining steady-state MT levels, as well as in the re-growth of
acentrosomal MT arrays in interphase S2 cells. The fly genome encodes two different γ-tubulins
(Raynaud-Messina et al., 2004): γTub23C, the ubiquitous isotype that plays an important role in mitotic
spindle assembly (Sunkel et al., 1995), and γTub37C, which functions in female meiotic spindle
assembly (Tavosanis et al., 1997). Double γ-tubulin mutants are severely compromised in various stages
of female germ-cell differentiation (Tavosanis and Gonzalez, 2003). Surprisingly, we found that co-
depletion of both γTub23C and γTub37C did not inhibit production of interphase MT arrays or alter
steady-state MT levels. Our findings differ from γ-tubulin loss-of-function in other systems, which
generally display severe changes in MT levels. For example, γ-tubulin RNAi in C. elegans embryos
inhibits the formation of interphase centrosomal asters and reduces MT levels by 60% during mitosis
(Hannak et al., 2002). In the fungi Ustilago maydis and Aspergillus nidulans, loss of γ-tubulin function
results in the reduction of cytoplasmic MT number and length (Straube et al., 2003; Oakley et al., 1990).
In contrast, conditional γ-tubulin mutants in Saccharomyces cerevisiae produced fewer nuclear/spindle
MTs and abnormally long cytoplasmic MTs (Marschall et al., 1996; Spang et al., 1996).

Acentrosomal MT nucleation is observed in plants and S. pombe (Bartolini and Gundersen, 2006), in
human cells from the Golgi apparatus (Efimov et al., 2007), and interphase MT arrays can self-organize
in anucleate fission yeast cells lacking centrosomes (Carazo-Salas and Nurse, 2006; Daga et al., 2006). Significantly, however, acentrosomal MT nucleation and organization in these systems differs from *Drosophila* cells, in that γ-tubulin is required for MT nucleation (Bartolini and Gundersen, 2006).

Although γ-tubulin does not appear to play an essential role in maintaining interphase steady-state MT levels in S2 cells, MT re-growth experiments revealed that γ-tubulin does participate in MT nucleation. In the absence of γ-tubulin, S2 cells subjected to cold-induced MT-depolymerization exhibit a delay in the full recovery of interphase MTs by 15 min; taking twice as long as controls. Similarly, γ-tubulin siRNA in cultured human cells, that normally use centrosomes to nucleate MTs, produced extensive acentrosomal MT arrays after a slight delay in MT re-growth (Lüders et al., 2006), although neither the extent of γ-tubulin depletion nor a measurement of MT levels were determined. Perhaps a γ-tubulin-independent mechanism for MT nucleation is conserved in animal cells (Lüders et al., 2006).

We hypothesize that γ-tubulins function redundantly with an unidentified MT-assembling factor to nucleate acentrosomal MTs. Consistent with this hypothesis, a recent genome-wide RNAi screen in S2 cells identified many genes involved in mitotic spindle assembly, but depletion of only a few individual genes disrupted MT assembly. These were mainly the α/β tubulins and tubulin folding proteins (Goshima et al., 2007). To identify redundant molecules, we examined interphase MT arrays in the absence of MAST/Orbit, CLIP-190, MspS, EB1, DHC or p150Glued, some of which possess MT-nucleating activity (Efimov et al., 2007; Malikov et al., 2004; Slep and Vale, 2007). Only MAST or MspS RNAi substantially reduced steady-state MT levels, potentially via their role in MT stability. Additionally, in most cases, MT levels were not further reduced by simultaneous depletion of γ-tubulin suggesting that none of these candidates acts redundantly with γ-tubulin to maintain the steady-state level of interphase MT arrays.

However, depletion of γ-tubulin, CLIP-190, MspS, EB1, or DHC all produce a 15 min delay in MT recovery, that is not further prolonged if γ-tubulin is co-depleted with any one of the remaining assembly factors. Thus, our findings suggest a model in which interphase *Drosophila* cells use a biphasic pathway
to re-establish acentrosomal MT arrays. One speculative possibility is that a multi-component ‘functional pathway’ of γ-tubulin/CLIP-190/Msps/EB1/DHC collectively drives an initial fast phase of MT nucleation. Theoretically, tubulin heterodimers bound to CLIP-190 and Msps could be recruited, via EB1, to newly formed MT ‘seeds’, unstable intermediate assembly-structures that are partially stabilized by binding DHC. A CLIP-190/Msps/EB1 complex could thus provide additional subunits to elongate a MT seed and also stabilize the formation of a nascent MT plus-end. Additional stability could then be provided by γ-tubulin capping a newly formed minus-end. Removal of any one of these critical functions would compromise seed stability and MT growth. Obviously, the exact mechanism of MT nucleation for this pathway is unclear as we lack a complete understanding of the MT stabilizing activity for any one of these proteins. Further, additional components may exist.

Interestingly, inhibition of the fast phase reveals the presence of a slower phase of MT nucleation that provides recovering S2 cells with a redundant mechanism to rebuild the interphase MT array. This back-up mechanism potentially explains why depletion of γ-tubulin has little effect on maintaining steady-state MT levels. The mechanism of slow phase MT nucleation is unknown but could be accomplished by MT self-assembly, an inherent property of αβ tubulin, or, perhaps, additional MT nucleators may remain to be discovered. Alternatively, the multi-component pathway that we describe could simply allow for more efficient MT assembly, whereby MTs still assemble after inactivation of the pathway, albeit more slowly, but not necessarily in a biphasic way.

Our data are consistent with MT nucleation at specific sites within the cell, as we observed γ-tubulin-independent formation of small MT foci during the course of a MT re-growth assay, resembling the MTs reported in other cold-treated cultured fly cells (Cottam et al., 2006). The Golgi serves as a non-centrosomal surface for MT nucleation in mammalian cells (Chabin-Brion et al., 2001; Efimov et al., 2007). In S2 cells, many of the MT foci are not associated with Golgi, suggesting that they are not the sole source of non-centrosomal MT nucleation. It is possible that MT nucleation occurs off of another membrane-bound organelle, such as the endoplasmic reticulum, that is normally dispersed in the
cell through its association with a pre-existing MT network. When MTs are depolymerized, this organelle might retract into a compact structure, with MT foci appearing at this site.

Taken together, our results support a canonical *Drosophila* cycle of centrosome function that differs from the canonical model in vertebrate cells and illustrates the flexibility of this functional cycle as well as the plasticity of the mechanisms for MT nucleation.

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**REFERENCES**


FIGURE LEGENDS

Figure 1. S2 cells lack a γ-tubulin-containing MTOC during interphase.

(A-F) S2 cells stained for MTs (red), γ-tubulin (green) and phosphohistone H3 (blue). Representative cells in early prophase (A), metaphase (B,E), telophase (C) and interphase (D,F). (E,F) γ-tubulin-GFP (green)-expressing stable line. Scale 5μm.

Figure 2. Drosophila embryos lack a γ-tubulin-containing MTOC during interphase. An embryo in the process of dorsal closure was fixed and stained for α-tubulin (A) and γ-tubulin (B). Inset shows embryo model with red box showing relative orientation of images in A and B. Three different cell populations are boxed in (A) corresponding to G2-arrested amnioserosa cells (1), G1 arrested-leading edge cells (2) and epithelial cells undergoing mitosis (3). Boxed regions are displayed at higher magnification. Insets show a bipolar spindle from a mitotic domain of a younger developing embryo with γ-tubulin labeling at the poles.

Figure 3. Centrioles selectively recruit γ-tubulin during mitosis but not interphase.

GFP-SAS-6 (green) expressing S2 cells in metaphase (A) or interphase (B) stained for γ-tubulin (red) and phosphohistone H3 (blue). GFP-SAS-6 localizes to a small spot inside a ring of γ-tubulin during mitosis (A, insets). 100% of mitotic γ-tubulin ‘spheres’ contained GFP-SAS-6 (n = 100). Scale 5μm. Embryonic leading edge cells (C), dividing epithelial cells (D), amnioserosa cells (E), and an embryonic dividing neuroblast (E), stained for D-PLP (green), γ-tubulin (red) and actin (blue). Yellow arrowheads mark centriole positions. Cell borders are traced (blue). Scale 10μm. Transmission electron micrographs of S2 cells during mitosis (G) and interphase (H, I). A dense MT array radiates from a centriole pair (yellow arrowhead) during prophase (G) (N, nucleus), higher magnification shown in G'. MTs (black arrowheads) do not emanate from centrioles during interphase (H, I), higher magnifications shown in H' and I'.
Figure 4. Interphase microtubule nucleation is independent of centrioles and γ-tubulin.

(A-F) Projections of GFP-SAS-6 (red) and EB1-mRFP (green) MT growth patterns visualized by merging short segments of a 1 minute time-lapse recording into a single flattened image. Tracks of SAS-6 spots in the panels correspond to their movement over time. Acentriolar MT nucleation occurs in interphase S2 cells (A) but is associated with centrioles during mitosis (B). In embryos, MT nucleation (EB1-GFP; green) is not associated with centrioles (mCherry-SAS-6; red) in interphase amnioserosal (C) or leading edge (D) cells but is associated with spindle poles in mitotic domains (E). White dotted-line (C) traces the cell-border. (F) A similar pattern of MT growth is observed after γTub23C RNAi. Scale 5μm (A,B,F), 10μm (C-E).

Figure 5. RNAi-mediated co-depletion of γTub23C/37C does not affect steady-state MT levels in interphase S2 cells. (A) Immunofluorescence of interphase and mitotic transfected S2 cells expressing γTub37C-GFP (green) and stained for D-PLP (red) and DNA (blue). The right spindle pole in the mitotic cell is not in the focal plane. Arrowheads mark centriole positions. (B) Lysates were prepared from γ-Tub37C-GFP stable-expressing S2 cells after 7 days of control or γ-tubulin RNAi-treatments and probed by Western blot using anti-γ-tubulin antibody (anti-α-tubulin was used as a loading control). (C) Bar graph shows the frequency of monopolar spindle formation after 7-day control, γ-tubulin or MAST RNAi-treatments and identified by MT staining (inset; scale 5μm). (D) Interphase day-7 control and γTub23C/37C RNAi-treated S2 cells stained for MTs. (E) Distribution histograms of total integrated MT fluorescence intensity in RNAi-treated α-tubulin-immunolabeled S2 cells using quantitative HTM; (y-axis) cell number, (x-axis) arbitrary units. Examples of these cells are shown in D. γ-tubulin RNAi treatments produced no significant difference (P > 0.05) as compared to control RNAi using a non-parametric Kruskal-Wallis one-way analysis of variance followed by a Dunn’s post test.

Figure 6. MT re-growth occurs independently of centrioles but is delayed in γ-tubulin depleted S2 cells. (A) S2 cells were stained for MTs (green), D-PLP centrioles (red) and DNA (blue) at specific time-
points in a MT re-growth assay. MTs were depolymerized by cold-treatment (0 min.) and brought to room temperature to allow polymerization. Cells were fixed at 5, 10 and 15 minutes. Centrioles (white arrowheads) are shown at higher magnifications in bottom panels (left to right; MTs, D-PLP, merge). A cell in cytokinesis is shown at 5 minutes with disengaged centrioles (insets). An interphase cell at 5 minutes contains individual MTs (yellow arrowheads) and tufts of MT foci (yellow arrow). Scale, 10μm. (B) Mitotic (top) and interphase (bottom) S2 cells after 5 minutes of MT re-growth stained for MTs (green), γ-tubulin (red, insets), and DNA (blue). The white box (interphase cell) is shown at higher magnification (lower panel) and denotes a cluster of MT foci (left to right; MTs, γ-tubulin, merge). (C) An S2 cell at 5 minutes of MT re-growth immunostained for MTs (green), Golgi (red), and DNA (blue). Higher magnifications of (1) individual MTs in the lamella, (2) MT foci, (3) Golgi associated with MT foci, and (4) Golgi punctae along a MT bundle. Scale, 10μm. (D) Time-points show day 7 control and γ-Tub23C/37C RNAi-treated S2 cells stained for MTs during MT re-growth. Scale, 10μm. (E) Quantitation of MT recovery in the RNAi-treated cells displayed in D.

Figure 7. CLIP-190 or Msps RNAi delays MT re-growth in S2 cells but is not observed with either MAST RNAi or co-depletion with γ-tubulin. (A) Distribution histograms of total integrated MT fluorescence intensity at steady-state in 5,000 RNAi-treated α-tubulin-immunolabeled S2 cells using quantitative HTM; (y-axis) cell number, (x-axis) arbitrary units. Approximately 5,000 cells were scanned in each treatment which produced a significant difference (P < 0.05) as compared to the control RNAi using a non-parametric Kruskal-Wallis one-way analysis of variance followed by a Dunn’s post test. (B) Time-points show representative day 6 RNAi-treated S2 cells stained for MTs during MT re-growth. Each row of micrographs is aligned with the dsRNA(s) that were used in A. Scale, 10μm.

Figure 8. EB1 or cytoplasmic dynein (DHC) RNAi delays MT re-growth in S2 cells but is not observed with either depletion of the p150Glued component of dynactin or co-depletion with γ-tubulin. (A) Distribution histograms of total integrated MT fluorescence intensity at steady-state in RNAi-treated α-
tubulin-immunolabeled S2 cells using quantitative HTM; (y-axis) cell number, (x-axis) arbitrary units. Approximately 5,000 cells were scanned in each treatment which produced a significant difference (P < 0.05) as compared to the control RNAi using a non-parametric Kruskal-Wallis one-way analysis of variance followed by a Dunn’s post test. (B) Time-points show representative day 6 RNAi-treated S2 cells stained for MTs during MT re-growth. Each row of micrographs is aligned with the dsRNA(s) that were used in A. Scale, 10μm.
SUPPLEMENTARY DATA

**Supplemental Figure 1.** Interphase day-7 control and γ-Tubulin23C RNAi-treated S2 cells stained for MTs (green), γ-tubulin (red), and DNA (blue). Scale, 5μm.

**Supplemental Figure 2.** *Drosophila* D16 cells lack a γ-tubulin-containing MTOC during interphase. Interphase (A) and mitotic (B) D16 cells were plated on concanavalin A and processed for immunofluorescence to reveal MTs (red), γ-tubulin (green) and DNA (blue). Scale, 5μm.

**Supplemental Figure 3.** (A) *Drosophila* SAS-6 co-localizes with the centriolar protein D-PLP. An interphase GFP-SAS-6 (green) expressing stable S2 cell stained for D-PLP (red) and DNA (blue). Magnified views of the centrioles are shown in insets. (B) Centrioles in *Drosophila* D16 cells co-localize with γ-tubulin in mitosis but not during interphase. D16 cells were plated on concanavalin A and processed for immunofluorescence with antibodies against γ-tubulin (red), D-PLP (green) and stained for DNA (blue). The panels illustrate typical cells in interphase and mitosis. Magnified views of the centrioles are shown in insets. Scale 5μm.

**Supplemental Figure 4.** The centrosomal proteins Centrosomin (Cnn), CP190, and CP60 localize to the PCM during mitosis but do not localize to centrioles during interphase. Interphase and mitotic stable S2 cells expressing GFP-SAS-6 (green) were fixed and stained for DNA (blue) and Cnn (red, top row), CP190 (red, middle row), or CP60 (red, bottom row). *Top panel,* the mitotic cell shown is in prophase. Magnified views of the centrioles and Cnn (white arrowheads) are shown below the micrographs. *Middle and bottom panels,* both CP190 and CP69 are restricted to the nucleus during interphase (DNA not shown), but are released into the cytoplasm during mitosis and concentrate on centrosomes at spindle poles (white arrowheads) (GFP-SAS-6 centrioles not shown for CP60). Magnified views of CP190-labeled mitotic centrosomes are shown in insets. Scale 5μm.
Supplemental Figure 5. *Drosophila* SAS-6 RNAi eliminates centrioles without altering interphase cell cycle progression. (A) Cells were fixed and stained for SAS-6 (green), γ-tubulin (red), and DNA (blue). Anti-SAS-6 antibody labels spots within γ-tubulin-stained rings at the spindle pole. Note a cluster of centrosomes at the right spindle pole (left pole is in a different focal plane). (B) S2 cell lysates were prepared prior to and 7 days after SAS-6 RNAi-treatment and probed by Western blot using anti-SAS-6 antibody (α-tubulin was used as a loading control). (C) Day 7 control and SAS-6 RNAi-treated S2 cells stained for D-PLP (red) and DNA (blue). White traces outline the cells. (D) Histogram of centrioles per interphase cell counted in control or SAS-6 RNAi-treated S2 cells. (E) SAS-6 RNAi reduces mitotic centrosome number. Control and SAS-6 RNAi cells were stained for γ-tubulin (red), α-tubulin (green) and DNA (blue). Percentages of spindles types are indicated. Scale, 2.5µm. (F) Cell cycle progression is unaffected by SAS-6 RNAi. Histograms of DNA fluorescence intensity (x-axis) and cell number (y-axis) of 7,000 cells with 2C, 4C and 8C populations denoted.

Supplemental Figure 6. Early periods of MT re-growth occur independently of centrioles and Golgi but both organelles can associate with MTs. S2 cells were stained for MTs (green) and either (A) D-PLP centrioles (red) or (B) Golgi (red) at specific early time-points in a MT re-growth assay. MTs were depolymerized by cold-treatment (0 min.) and brought to room temperature to allow polymerization. Cells were fixed at 0, 2.5 and 5 minutes. (A) Centrioles are numbered and shown at higher magnifications (insets). At 2.5 minutes, MTs assemble but most are not associated with centrioles. At 5 minutes, MT foci form, most of which do not contain centrioles (1), although some MT foci do co-localize with centrioles (2). (B) Golgi are numbered and shown at higher magnifications (insets). At 2.5 and 5 minutes, MTs assemble as small (2.5 min.) and larger (5 min.) foci, most of which are not associated with Golgi (1). However, some MT foci do associate with Golgi at these time-points (2). Scale, 5µm.
Supplemental Figure 7. Interphase day-7 control, MAST, and Mini-spindles (Msps) RNAi-treated S2 cells stained for MTs (the cell margin for the MAST depleted cell is traced in white). Scale, 5μm.

Supplemental Figure 8. The kinetics of MT re-growth is not altered in either Ncd, Klp10A, Shortstop (Shot), or Kinesin heavy chain (KHC)-depleted S2 cells. Time-points show representative day 6 RNAi-treated S2 cells stained for MTs during MT re-growth. Scale, 5μm.

MOVIE LEGENDS

Movie 01. Interphase microtubule nucleation is independent of centrioles.
Expression of EB1-mRFP (green) allows the visualization of microtubule nucleation and growth relative to a GFP-SAS-6-labeled centriole (red) in a live interphase S2 cell.

Movie 02. Mitotic microtubule nucleation is associated with centrioles.
Expression of EB1-mRFP (green) allows the visualization of microtubule nucleation and growth relative to GFP-SAS-6-labeled centrioles (red) in a live prophase S2 cell.

Movie 03. Interphase microtubule nucleation is independent of centrioles in live embryonic amnioserosal cells.
Expression of EB1-GFP (green) allows the visualization of microtubule nucleation and growth relative to mCherry-SAS-6-labeled centrioles (red) in live embryonic amnioserosal cells. These differentiated cells are terminally-arrested in interphase (G2 phase). The central cell in this movie has an elongated-diamond shape. Centrioles display wide oscillations in their movements throughout the cell.

Movie 04. Interphase microtubule nucleation is independent of centrioles in live embryonic leading edge cells.
Expression of EB1-GFP (green) allows the visualization of microtubule nucleation and growth relative to mCherry-SAS-6-labeled centrioles (red) in live embryonic leading edge cells. These differentiated cells are terminally-arrested in interphase (G1 phase), and were observed during dorsal closure. The
direction of leading edge cell migration occurs toward the top of the image. Several leading edge cells are seen in this field of view and each has an elongated morphology.

**Movie 05.** Mitotic microtubule nucleation is associated with centrioles in live embryonic dividing epithelial cells. Expression of EB1-GFP (green) allows the visualization of microtubule nucleation and growth relative to mCherry-SAS-6-labeled centrioles (red) in live embryonic dividing epithelial cells. Several metaphase-stage spindles are seen within this region of a stage 9 embryo with centrioles at the spindle poles associated with the brightest EB1 signal.

**Movie 06.** Interphase microtubule nucleation is independent of both centrioles and γTub23C. Expression of EB1-mRFP (green) allows the visualization of microtubule nucleation and growth relative to GFP-SAS-6-labeled centrioles (red) in a live 7-day RNAi-treated interphase S2 cell depleted of the major γTub23C isotype. Two centrioles move throughout this cell and are not associated with the microtubule nucleation that appears indistinguishable relative to control cells (see Movie 01). Centrioles are sometimes seen moving in fast linear trajectories which presumably occurs along MT tracks.
A

Control RNAi
Mean 81,623
Median 74,681

MAST
Mean 53,465
Median 43,405

MAST/γTub
Mean 43,084
Median 34,503

CLIP-190
Mean 87,429
Median 78,278

CLIP-190/γTub
Mean 81,740
Median 71,246

MspS
Mean 46,534
Median 37,496

MspS/γTub
Mean 46,853
Median 37,303

Total Integrated
Anti-α-Tubulin
Fluorescence Intensity

B

0 min 5 min 10 min 15 min

30 min