Stathmin Regulates Centrosomal Nucleation of Microtubules and Tubulin Dimer/Polymer Partitioning

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Stathmin is a microtubule-destabilizing protein ubiquitously expressed in vertebrates and highly expressed in many cancers. In several cell types, stathmin regulates the partitioning of tubulin between unassembled and polymer forms, but the mechanism responsible for partitioning has not been determined. We examined stathmin function in two cell systems: mouse embryonic fibroblasts (MEFs) isolated from embryos +/+, +/-, and -/- for the stathmin gene and porcine kidney epithelial (LLCPK) cells expressing stathmin-cyan fluorescent protein (CFP) or injected with stathmin protein. In MEFs, the relative amount of stathmin corresponded to genotype, where cells heterozygous for stathmin expressed half as much stathmin mRNA and protein as wild-type cells. Reduction or loss of stathmin resulted in increased microtubule polymer but little change to microtubule dynamics at the cell periphery. Increased stathmin level in LLC PK cells, sufficient to reduce microtubule density, but allowing microtubules to remain at the cell periphery, also did not have a major impact on microtubule dynamics. In contrast, stathmin level had a significant effect on microtubule nucleation rate from centrosomes, where lower stathmin levels increased nucleation and higher stathmin levels reduced nucleation. The stathmin-dependent regulation of nucleation is only active in interphase; overexpression of stathmin-CFP did not impact metaphase microtubule nucleation rate in LLC PK cells and the number of astral microtubules was similar in stathmin +/+ and -/- MEFs. These data support a model in which stathmin functions in interphase to control the partitioning of tubulins between dimer and polymer pools by setting the number of microtubules per cell.

INTRODUCTION

Microtubules (MTs) are dynamic polymers composed of α/β -tubulin heterodimers that in cells exist at steady state with a pool of unassembled tubulin dimers. In a nondifferentiated cell, ~65% of tubulins are assembled into MTs and 35% are present as dimers (Zhai *et al.*, 1996). This partitioning of tubulin dimers between polymer and dimer pools is present in both interphase and metaphase of mitosis (Zhai *et al.*, 1996), although the lengths, number, and dynamic turnover rates of the MTs are very different between these two cell cycle states (Desai and Mitchison, 1997). Cells are also able to regulate the partitioning of tubulins between soluble and polymer pools, for example, briefly shifting most tubulins into the dimer pool at entry into mitosis (Zhai *et al.*, 1996) or shifting dimers into polymer form in T cells responding to signals downstream of the T cell receptor/CD3 complex (Holmfeldt *et al.*, 2007).

Several potential mechanisms could regulate partitioning of tubulin between dimer and polymer pools. First, shifts in the plus end dynamic instability of MTs, favoring net assembly or disassembly, could lead to changes in the partitioning of tubulins. By this mechanism, changes in the level or activity of microtubule-associated proteins regulating various parameters of dynamic instability would alter tubulin dimer partitioning. Such a shift in tubulins from polymer

to dimers was observed after depletion of microtubule-associated protein (MAP) 4, an MT-stabilizing protein (Nguyen *et al.*, 1999). A second factor possibly regulating dimer/polymer partitioning is the rate of MT nucleation from centrosomes. Several computational models have suggested that MT nucleation rate could have a significant impact on tubulin partitioning within a cell (Mitchison and Kirschner, 1987; Gregoretti *et al.*, 2006), although this has not been tested experimentally. Additional mechanisms that could contribute to tubulin partitioning between dimer and polymer pools include regulation of the total tubulin level or sequestration of tubulin dimers to render them unable to polymerize (Mitchison and Kirschner, 1987; Holmfeldt *et al.*, 2007; Sellin *et al.*, 2008).

One MT regulatory protein recently demonstrated to contribute to tubulin dimer/polymer partitioning is stathmin (also named oncoprotein 18; gene name *Stmn1*), an 18-kDa phosphorylation-regulated MT-destabilizing protein expressed ubiquitously in vertebrates (Steinmetz, 2007) and overexpressed in a wide range of cancers (Brattsand *et al.*, 1993; Curmi *et al.*, 2000; Mistry *et al.*, 2005). The role of stathmin in regulating tubulin dimer/polymer partitioning was shown by overexpression or microinjection of stathmin in vertebrate cells, which reduced MT polymer dramatically (Larsson *et al.*, 1999; Wittmann *et al.*, 2004; Holmfeldt *et al.*, 2007), whereas microinjection of anti-stathmin antibodies (Howell *et al.*, 1999a) or short hairpin RNA-based depletion of endogenous stathmin (Holmfeldt *et al.*, 2006; Sellin *et al.*, 2008) led to increased MT polymer. Stathmin-dependent regulation of MT polymer level is not confined to cells in culture because stathmin knockout mice have more MT polymer in the amygdala region of the brain compared with wild-type mice (Shumyatsky *et al.*, 2005).

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Abbreviations used: MEF, mouse embryo fibroblast; MT, microtubule; *Stmn1*, stathmin.

Stathmin could contribute to tubulin dimer/polymer partitioning through several possible mechanisms. Each stathmin molecule can bind and sequester two tubulin dimers, thus preventing their polymerization (Howell *et al.*, 1999b; Steinmetz, 2007). A significant sequestering function is possible in those cells where stathmin concentration nears that of tubulin, such as Jurkat or K562 leukemia cells (Sellin *et al.*, 2008). In Jurkat cells (Sellin *et al.*, 2008) and *Drosophila* embryos (Fletcher and Rorth, 2007), stathmin also contributes to regulation of the total tubulin level, which may contribute to dimer/polymer partitioning. Stathmin also functions independently of tubulin sequestration to stimulate MT dynamics by increasing catastrophes at MT plus ends (Belmont and Mitchison, 1996; Howell *et al.*, 1999b), which could also contribute to tubulin dimer partitioning (Howell *et al.*, 1999a).

Here, we use two cell systems derived from noncancerous cells, which express relatively low levels of stathmin, to explore how stathmin regulates tubulin dimer partitioning in the absence of significant tubulin sequestering activity. We isolated mouse embryonic fibroblasts (MEFs) from embryos wild-type (stathmin^{+/+}), heterozygous (stathmin^{+/-}), and null (stathmin^{-/-}) for the stathmin gene. We also transiently overexpressed stathmin in porcine kidney epithelial (LLCPK) cells. For both cell types, we measured MT plus-end dynamics and nucleation rates. Our results support a model in which stathmin regulates tubulin partitioning by regulating the rate of MT nucleation, rather than by regulating MT dynamics at the cell periphery.

MATERIALS AND METHODS

Isolation of MEFs

C57BL/6 Stmn1^{+/-} male and female mice (a generous gift from G. Shumyatsky, Rutgers University) were mated. Pregnant females were killed by cervical dislocation 13.5 to 14.5 d after coitus, and fibroblasts were isolated as described by Tessarollo (2001). Fibroblast cells from individual embryos were plated and allowed to grow for 1–3 d before storage of aliquots in liquid nitrogen.

Genotyping of MEFs was performed as described by Liedtke *et al.* (2002). In brief, DNA was isolated from embryonic tissue by using isoamyl alcohol/phenol extraction methods. Samples were amplified using polymerase chain reaction (PCR) to identify embryos with intact Stmn1 intron III or the neomycin cassette used to disrupt the Stmn1 gene (Schubart *et al.*, 1996). The PCR Jump Start REDTaq kit (Sigma-Aldrich, St. Louis, MO) was used for amplification; each sample contained 1.5 mM MgCl₂, deoxynucleotide triphosphates (200 μ M each), primers (0.5 μ M each), and Taq polymerase (0.05 U/ μ L). For the wild-type amplification, 35 temperature cycles were performed (95°C, 35 s; 66°C, 45 s; and 72°C, 45 s). Primers used for genotyping are listed in Supplemental Table 1; these primers amplify either a portion of the region of intron III deleted in the mutant allele or mutant-allele-specific primers that recognize a portion of the neomycin insert (Schubart *et al.*, 1996).

Cell Culture

All cells were cultured at 37°C in a humidified atmosphere of 5% carbon dioxide. Cells were grown in DMEM, pH 7.4, supplemented with 4.5 g/L D-glucose, L-glutamine (Invitrogen, Carlsbad, CA), 44 mM sodium bicarbonate, antibiotic/antimycotic (Sigma-Aldrich), 1 mM sodium pyruvate (Sigma-Aldrich), and 10% fetal bovine serum (Invitrogen). LLCCK GFP fluorescent protein (GFP)-tubulin (Rusan *et al.*, 2001) and LLCCK GFP-EB1 (Piehl and Cassimeris, 2003) cell lines were maintained as described previously (Piehl and Cassimeris, 2003). MEF cultures were discarded after passage 7.

Microinjection

LLCPK GFP- α -tubulin cells were microinjected with bacterially expressed stathmin-FLAG (130 μ M stock concentration; Holmfeldt *et al.*, 2006) as described previously (Piehl and Cassimeris, 2003). Injected cells were marked by coinjection of Alexa-Fluor 594-conjugated bovine serum albumin. Others have estimated microinjection volumes of ~2.5–10% of total cell volume (Graessmann *et al.*, 1980; Saxton *et al.*, 1984; Howell *et al.*, 1999a, 2000), indicating that intracellular concentration of stathmin-FLAG was ~3–13 μ M.

Transient Transfection

A plasmid for expression of stathmin-cyan fluorescent protein (CFP) was constructed by amplifying the human stathmin cDNA from a pBS-STMN plasmid (provided by Martin Gullberg) and introducing HindIII and BamHI restriction sites by using primers listed in Supplemental Table 1. The reverse primer also removed the stop codon. The HindIII/BamHI fragment was ligated into pECFP-N1 and pEGFP-N1 (Clontech, Mountain View, CA). Cells were transfected with plasmids encoding stathmin-GFP/CFP, GFP-tubulin, or EB1-GFP as described previously (Piehl and Cassimeris, 2003; Warren *et al.*, 2006).

Indirect Immunofluorescence

Fixed cells were analyzed by immunofluorescence as described previously (Piehl and Cassimeris, 2003). Antibodies used included mouse monoclonal antibody to α -tubulin (B512; Sigma-Aldrich), rabbit anti- γ -tubulin (Sigma-Aldrich), mouse anti-EB1 (BD Biosciences, San Jose, CA), and goat-anti-mouse or anti-rabbit Alexa Fluor 488 or 563 (Invitrogen). In some experiments, DNA was also labeled with TO-PRO-3 iodide (Invitrogen).

Protein Isolation and Western Blot Analysis

Cell lysates or fractions from cytoskeletal and supernatant fractions were prepared for SDS-polyacrylamide gel electrophoresis as described previously (Graessmann *et al.*, 1980; Saxton *et al.*, 1984; Minotti *et al.*, 1991; Howell *et al.*, 1999a). Protein concentrations of soluble fractions were determined by Bradford assay (Bradford, 1976). Membranes were probed with anti-tubulin antibodies DM1A or Tub2.1, or anti-stathmin (Sigma-Aldrich) followed by goat anti-rabbit or mouse horseradish peroxidase-linked immunoglobulin G (Sigma-Aldrich). Enhanced chemiluminescence (PerkinElmer Life and Analytical Sciences, Boston, MA) was used to develop immunoreactive bands according to manufacturer's specifications. For semiquantitative estimates of stathmin and tubulin concentrations, band intensities from cell lysates were compared with intensities for purified porcine brain tubulin (purified as described in Vasquez *et al.*, 1994) and probed with Tub2.1 anti- β -tubulin or bacterially expressed stathmin-FLAG (Holmfeldt *et al.*, 2006; a generous gift from Martin Gullberg).

Confocal Microscopy

Confocal microscopy was used to image fixed and living cells as described previously (Warren *et al.*, 2006). To image GFP-tubulin for MT dynamics or EB1-GFP to measure MT nucleation rates, 30–35 images were acquired every 4 s by using two-line mean averaging, requiring a scan time of 3.5–4.0 s. To count all EB1-GFP comets per cell, Z series were collected from live LLCCK-EB1-GFP cells with or without stathmin-CFP expression. Z series also were collected for fixed mitotic MEFs labeled with antibodies to EB1.

Image Analysis and Microtubule Tracking

MetaView imaging software (Molecular Devices, Sunnyvale, CA) was used to measure polymer level in MEFs, as described previously (Howell *et al.*, 1999a), for cells fixed in ice-cold methanol and stained for α -tubulin. MetaView software also was used to track length changes of individual MTs in cells expressing GFP- α -tubulin as described previously (Piehl and Cassimeris, 2003; Warren *et al.*, 2006). Interphase MTs were selected for tracking if they had clearly discernible plus-end tips at the cell periphery that persisted for at least 30 frames, or 120 s. All clearly defined MTs within an image series were tracked. Images acquired from LLCCK-GFP-tubulin cells expressing CFP or stathmin-CFP were later used to measure MT density at the cell periphery by counting the number of MTs within 5 μ m of the cell cortex. MetaMorph imaging software (Molecular Devices) was used to manually count all EB1-GFP comets per cell from Z-series image stacks.

Dynamic Instability Calculations

Parameters of dynamic instability were calculated as described previously (Warren *et al.*, 2006; Warren and Cassimeris, 2007). Rates of growth and shortening, and total time spent in growth, pause, or shortening were calculated for each MT tracked. Dynamicity was calculated by summing the total lengths of MT polymer gained and lost for each MT divided by the total time observed (Warren *et al.*, 2006). Dynamicity describes total gain and loss of tubulins per unit time. Drift velocity, a measure of net growth or shortening, was calculated as described previously (Vorobjev *et al.*, 1999; Warren *et al.*, 2006). The frequencies of catastrophe (k_{cat}) and rescue (k_{res}), were calculated as described by Rusan *et al.* (2001). The standard deviations for transition frequencies were calculated from catastrophe frequencies, or rescue frequencies, divided by the square root of the number of transitions observed (Walker *et al.*, 1988; Howell *et al.*, 1999b). Statistical analyses were performed by single-factor analysis of variance (ANOVA) in Excel (Microsoft, Redmond, WA) or Student's *t* test (Howell *et al.*, 1999a).

RNA Isolation

Total RNA was isolated from cultured MEF cell lines by using TRIzol reagent (Invitrogen) following manufacturer's instructions, followed by DNase treat-

Figure 1. Stathmin genotypes and protein levels in MEF lines. (A) Representative PCR amplicons are shown for each genotype. Genotypes of embryos and cell lines were assigned based on the presence or absence of the *Stmn1* gene and the neomycin gene used to disrupt the *Stmn1* gene. (B) qRT-PCR of stathmin mRNA derived from each cell line, shown as $\pm$ -fold change relative to stathmin^{+/+}. Stathmin mRNA is reduced by 50% in stathmin^{+/-} cells and was not detectable in stathmin^{-/-} cells. (C) Stathmin protein level corresponds to the copy number of the *Stmn1* gene. (D) Semiquantitative estimate of stathmin concentration in wild-type MEFs. The left four lanes show decreasing loads of purified stathmin. The detection limit was $\sim$ 0.1 ng. Two stathmin^{+/+} MEF lines (3 μ g of total protein per lane) are shown relative to the stathmin standards. Based on immunoblot band intensities, these lines express $\leq$ 0.5 ng/3 μ g total protein.

ment. RNA was used to synthesize cDNA with SuperScript III First-Strand Synthesis System (Invitrogen) for quantitative reverse transcription-PCR (qRT-PCR).

qRT-PCR

Oligonucleotide probes were designed using Primer Express Software (Applied Biosystems, Foster City, CA; Supplemental Table 1) and cDNA sequences from the mouse genome (Benson *et al.*, 2007). Stathmin message levels were quantified for each genotype by using oligonucleotide probes designed to amplify cDNA fragments containing stathmin (Supplemental Table 1). Results were normalized to the signal from glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Amplification was achieved using Power SYBR Green PCR Master Mix (Applied Biosystems) and 7300 Real-Time PCR System (Applied Biosystems) with SDS version 1.4 software. RT-PCR amplicon specificity was checked by electrophoresis of RT-PCR products on 2% agarose gels (data not shown). Following manufacturer's instructions (Applied Biosystems), the threshold for determining C_T values was set to log scale 0.2, and internal normalization of genotype results to GAPDH was first calculated (i.e., C_T target mRNA $- C_T$ GAPDH mRNA = ΔC_T). ΔC_T values were not calculated for target mRNA samples that exceeded 35 cycles before crossing the threshold. The relative abundance of target mRNA between genotypes was calculated as 2 raised to the negative of the difference in ΔC_T values [i.e., $2^{-(\Delta C_T +/+ \text{ target mRNA} - \Delta C_T +/- \text{ or } -/- \text{ target mRNA})}$] (Applied Biosystems).

All statistical analyses were done using Excel (Microsoft). RT-PCR data are presented as $\pm$ -fold changes relative to MEF Stmn1^{+/+} samples. SEs were computed using ΔC_T values transformed to SE values of $\pm$ -fold change using the formula $2^{-(\Delta C_T +/+ \text{ target mRNA} - \Delta C_T +/- \text{ or } -/- \text{ target mRNA}) \pm SE \Delta C_T}$.

Significance was determined by ANOVA of ΔC_T values (Gründemann *et al.*, 2008; Westberry *et al.*, 2008). Amplification efficiency was determined to be 100% for GAPDH for each genotype per manufacturer's instructions (data not shown), and it was assumed that efficiency of target genes was also 100% for our statistical analysis.

RESULTS

Mouse Embryo Fibroblast Cell Lines Differing in Stathmin Genotype

To establish cell lines expressing varying levels of stathmin, we isolated MEF lines from mouse embryos (+/+), (+/-), and (-/-) for the *Stmn1* gene. The genotypes of the embryos and MEF lines were determined by PCR amplification of either a region of intron 3 present in wild-type but not in knockout lines, or a region of the neomycin cassette that replaced a portion of the *Stmn1* gene (Figure 1A and

Figure 2. MT polymer level increased with loss of stathmin. (A) Maximum intensity projections of MTs in MEF cell lines after fixation and staining for α -tubulin. Bar, 25 μ m. (B) Mean MT polymer content per cell $\pm$ SD averaged from three experiments. Both stathmin^{+/-} (n = 22) and stathmin^{-/-} (n = 19) lines contain more MT polymer than wild-type cells (**p < 0.001; n = 19). (C) Relative β -tubulin levels in MEF lines detected by immunoblots probed with a pan- β -tubulin antibody, Tub 2.1. Total tubulin remains nearly constant between cell lines differing in *STMN1* genotype. (D) Supernatant (S) and cytoskeletal pellet (P) fractions from stathmin^{+/+} and stathmin^{-/-} MEFs. Deletions of both copies of the stathmin gene shift almost all tubulin into polymer.

Supplemental Table 1) (Schubart *et al.*, 1996). *Stmn1* mRNA levels, measured by qRT-PCR, corresponded to the genotypes of the MEF lines: stathmin^{+/-} MEFs contained half as much stathmin mRNA as stathmin^{+/+} MEFs, whereas stathmin^{-/-} MEFs were void of stathmin mRNA (Figure 1B). At the protein level, stathmin^{+/-} MEFs expressed $\sim$ 50% of the stathmin of wild-type cells. Stathmin protein was undetectable in stathmin^{-/-} MEFs (Figure 1C). Measurements of stathmin mRNA and protein levels were consistent with those reported previously for mouse neonatal brain and 2-mo-old testicular tissues (Schubart *et al.*, 1996) and demonstrate that stathmin mRNA and protein expression are proportional to the stathmin genotype.

We also estimated the relative abundance of stathmin and tubulin in MEFs based on Western blots by comparing the band intensities from purified proteins or cell lysates. We estimate that the stathmin level in wild-type MEFs is $\sim$ 170 ng/mg total protein, or $\sim$ 0.017% of total cell protein (Figure 1D), consistent with previous measurements from nontransformed cells (120–330 ng/mg; Brattsand *et al.*, 1993). Blots also were probed with anti- β tubulin Tub2.1, a pan-tubulin antibody that recognizes all β -tubulin isotypes (Matthes *et al.*, 1988). The β -tubulin concentration in MEFs was $\sim$ 12 μ g/mg total protein (data not shown) and was similar in all three stathmin genotypes (Figure 2). Assuming that α - and β -tubulins are present at equal levels, we estimate that the tubulin concentration in each cell line is $\sim$ 24 μ g/mg total protein, or 2.4% of total protein, which is also consistent with previous estimates of tubulin concentration in cells ($\sim$ 2–3% of total cell protein; Hiller and Weber, 1978; Sellin *et al.*, 2008). Based on our estimates for tubulin and stathmin concentrations, tubulin is present at approximately a 25-fold molar excess over stathmin in wild-type MEFs and, therefore, at a 50-fold molar excess over stathmin in MEFs heterozygous for stathmin.

Stathmin Regulates Tubulin Dimer/Polymer Partitioning and MT Nucleation with Little Impact on MT Plus-End Dynamics

Stathmin regulation of tubulin dimer/polymer partitioning has been well documented in leukemia-derived lines (Marklund *et al.*, 1996; Holmfeldt *et al.*, 2002; Sellin *et al.*, 2008), but mechanisms responsible for shifts in tubulin dimer partitioning between soluble and polymer pools have not been fully explored. Stathmin also regulates MT plus end dynamics and polymer level, as shown in primary cultures of newt lung cells (Howell *et al.*, 1999a). Consistent with these previous studies, we found that MT polymer levels increased with stathmin level decrease, as shown by fluorescence intensity measurements of α -tubulin-stained cells (Figure 2, A and B). Stathmin^{+/-} cells had ~1.4 times the MT polymer of wild-type MEFs. Polymer level increased to 1.6 times the wild-type level in stathmin^{-/-} MEFs (Figure 2B), similar to the increases observed after transient knockdown or inhibition of stathmin (Holmfeldt *et al.*, 2007). The increase in MT polymer in stathmin^{+/-} and stathmin^{-/-} MEFs was not associated with an overall increase in the concentration of tubulin (Figure 2C). These data indicated that the soluble tubulin pool should be reduced in stathmin^{-/-} MEFs. To test this prediction directly, we separated soluble and cytoskeletal fractions from stathmin^{+/+} and stathmin^{-/-} MEFs. As shown in Figure 2D, loss of stathmin shifted most tubulin into the cytoskeletal fraction. A similar increase in MT polymer and decreased tubulin dimer pool was observed recently in K562 leukemia cells after transient knockdown of stathmin (Sellin *et al.*, 2008).

To determine the impact of stathmin on MT dynamics, MEF cell lines were transiently transfected with GFP- α -tubulin, and the dynamic instability of their individual MTs was tracked by time-lapse fluorescence microscopy at the cell periphery. In all three cell lines, MTs extended to the cell periphery and were dynamic. In general, MT dynamics were similar among cells from all three stathmin genotypes. Growth and shortening rates were both slightly reduced in stathmin^{-/-} cells compared with wild-type and stathmin^{+/-} cells. Other parameters of dynamic instability, including catastrophe and rescue frequencies, were similar in all three cell lines (Table 1). The similar parameters for dynamic instability in cells from all three stathmin genotypes is also shown by the relatively small differences in dynamicity (the total gain and loss of subunits per unit time) and drift velocity (the net gain or loss of subunits over time; Table 1). Although not significantly different, drift velocity was slightly negative in stathmin^{+/+} cells and shifted to slightly positive values in stathmin^{+/-} and stathmin^{-/-} cells, indicating that MT dynamics shift toward net growth in the absence of stathmin.

Differences in MT dynamics among MEFs of different stathmin genotypes were small compared with the changes in MT polymer level, indicating that stathmin could regulate polymer level through additional mechanisms. To probe whether stathmin contributes to MT nucleation rate from centrosomes, we counted new MTs as they emerged from the centrosome using GFP-EB1 comets to mark MT plus ends (Piehl *et al.*, 2004). Stathmin^{+/+} MEFs nucleated 14 ± 3 MTs/min (Figure 3A). Nucleation rate was higher in stathmin^{+/-} MEFs, although not statistically significant, and increased significantly to 20 ± 2 MTs/min in stathmin^{-/-} MEFs (Figure 3A).

Table 1. MT dynamic instability in MEFs of various stathmin genotypes

	MEF genotype		
	+/+	+/-	-/-
Total cells	9	5	8
Total MTs tracked	25	19	23
Total time tracked (s)	4126	2580	3210
Dynamic instability parameters			
Growth rate ($\mu\text{m}/\text{min}$)	7.1 ± 4.0	7.1 ± 3.3	$6.0 \pm 2.3^{**}$
Shortening rate ($\mu\text{m}/\text{min}$)	9.0 ± 5.4	8.3 ± 4.5	$7.4 \pm 5.2^*$
% time spent in growth	23.8	24.8	25.0
% time spent in shortening	17.2	17.2	15.8
% time spent in pause	37.4	58.1	48.8
Dynamicity ($\mu\text{m}/\text{min}$)	4.33 ± 3.06	3.07 ± 1.26	3.09 ± 1.63
Drift velocity ($\mu\text{m}/\text{min}$)	-0.07 ± 1.32	0.18 ± 0.73	0.43 ± 0.80
Catastrophe frequency (s^{-1})	0.042 ± 0.004	0.038 ± 0.004	0.035 ± 0.004
Rescue frequency (s^{-1})	0.054 ± 0.005	0.050 ± 0.005	0.065 ± 0.006

Confocal microscopy and subsequent image analysis were used to track changes in MT lengths and to quantify the various parameters of dynamic instability as described in *Materials and Methods*. Data are given as mean $\pm$ SD. ANOVA was used to determine significant differences (growth rate, shortening rate, dynamicity, drift velocity, nucleation rate). Two-tailed Student's *t* test was applied to catastrophe and rescue frequencies. **p* < 0.05 and ***p* < 0.01.

MT Dynamics and Nucleation in LLCPC Cells with Elevated Stathmin

Measurement of MT dynamics in MEF lines indicated that stathmin knockout had little impact on plus-end dynamic instability, whereas significantly increasing the amount of MT polymer. To confirm these results, we also examined MT dynamics and nucleation rate in LLCPC-GFP-tubulin epithelial cells transiently overexpressing stathmin-CFP. Our observations were confined to cells expressing moderate levels of stathmin-CFP, in which MTs were detectable. In

Figure 3. MT nucleation rates in MEFs and LLCPC cells. Nucleation rate was measured by counting the number of EB1-GFP comets emerging from the centrosome over time (Piehl *et al.*, 2004). (A) MT nucleation rate in MEFs increased in the absence of stathmin. (B) Expression of stathmin-CFP in LLCPC epithelial cells decreased nucleation rate. Data for A and B are means $\pm$ SD from five to 13 cells per condition. For each graph, **p* < 0.05 and ***p* < 0.01.

Table 2. Dynamic instability in LLC PK cells

	CFP control	Stmn-CFP	Stmn-Flag
Total cells	14	17	7
Total MTs tracked	49	60	44
Total time tracked (s)	6624	8120	5980
Dynamic instability parameters			
Growth rate ($\mu\text{m}/\text{min}$)	8.5 ± 5.8	7.9 ± 4.5	$7.3 \pm 3.3^*$
Shortening rate ($\mu\text{m}/\text{min}$)	11.3 ± 7.9	10.5 ± 8.0	$9.2 \pm 6.3^*$
% time spent in growth	40.4	36.8	27.0
% time spent in shortening	26.0	25.1	26.0
% time spent in pause	36.5	40.9	47.1
Dynamicity ($\mu\text{m}/\text{min}$)	5.3 ± 2.7	4.5 ± 3.2	$3.6 \pm 1.5^*$
Drift velocity ($\mu\text{m}/\text{min}$)	0.7 ± 1.6	0.2 ± 1.8	0.2 ± 1.0
Catastrophe frequency (s^{-1})	0.053 ± 0.003	0.044 ± 0.003	0.044 ± 0.003
Rescue frequency (s^{-1})	0.086 ± 0.005	$0.066 \pm 0.004^*$	$0.063 \pm 0.004^*$

MT dynamics were measured in LLC PK cells stably expressing GFP- α -tubulin and CFP or stathmin (stmn)-CFP. Additional cells were microinjected with stathmin-FLAG as described in *Materials and Methods*.

these stathmin-CFP-expressing cells, MT dynamics were similar to that measured in cells expressing CFP (Table 2). The only significant change was a reduced rescue frequency. All other parameters, including dynamicity and drift velocity, were not significantly different between cells expressing CFP or stathmin-CFP.

The expression of stathmin-CFP in LLC PK cells showed minor changes in MT dynamics compared with control cells, whereas at higher expression, reduced MT density made it difficult to image MTs. Attempts to estimate the level of stathmin-CFP expression indicated a wide distribution of expression levels (our unpublished observations), which prevented qualitative estimates of the amount of stathmin-CFP expression in those cells in which we measured MT dynamics. As an alternative, we also microinjected LLC PK-GFP-tubulin cells with purified stathmin-FLAG. Cells microinjected with stathmin-FLAG again showed MT dynamics similar to control cells (expressing CFP; Table 2), although growth and shortening rates were slightly slower and rescue frequency was reduced. Cells injected with stathmin-FLAG also showed reduced dynamicity. The small impact of stathmin on MT dynamics at the cell periphery was therefore not due to the tag on stathmin.

We next measured MT nucleation from the centrosome, because nucleation rate was sensitive to stathmin level in MEFs (Figure 3A). Expression of stathmin-CFP decreased nucleation rate by $\sim 40\%$ compared with that measured in cells expressing CFP (Figure 3B). The MT nucleation data are consistent with the results in MEFs, pointing to a role of stathmin in regulating MT nucleation from the centrosome.

We then asked whether stathmin-CFP or stathmin-FLAG, at the levels expressed in those cells having sufficient MTs for dynamics measurements, was able to modulate tubulin dimer/polymer partitioning. We used images previously collected for dynamics measurements and counted the number of plus ends extending to within $5 \mu\text{m}$ of the plasma membrane. By this measurement, MT density at the cell periphery dropped by $\sim 33\%$ for cells expressing stathmin-CFP compared with those expressing CFP and dropped by

Figure 4. MT density is reduced in cells having increased stathmin concentration. (A) MT density was measured by counting the number of MTs extending to within $5 \mu\text{m}$ of the cell membrane and shown as the number of MTs per $10 \mu\text{m}$ of cell circumference. Images used for measuring MT density were the same as those used for dynamics measurements (see Table 2). Examples of images (inverted gray scale) used to measure MT density are shown to the right. These images were selected initially only for dynamics measurements and the sole selection criterion was clarity of the MT ends. Data shown are means $\pm$ SD from 19 to 24 cells. Bar, $5 \mu\text{m}$. (B) The total number of growing MTs per cell was measured by counting all EB1-GFP comets per cell. Data shown are means $\pm$ SD from 21 to 22 cells. Examples of images (inverted gray scale) are shown to the right. Stathmin-CFP-expressing cell is marked by an arrow. The insets show enlarged regions from the cell periphery. Bar, $20 \mu\text{m}$. For both A and B, $**p < 0.01$.

50% in cells microinjected with stathmin-FLAG (Figure 4A). We confirmed these results by counting the total number of growing MTs per cell, marked by EB1-GFP comets, in either untransfected cells or cells expressing moderate levels of stathmin-CFP. As shown in Figure 4B, cells expressing stathmin-CFP had significantly fewer growing MTs per cell.

Stathmin Does Not Regulate MT Nucleation at Metaphase

The above-mentioned analyses focused on interphase cells because stathmin is thought to be inactivated in mitosis by phosphorylation of four serine residues (Larsson *et al.*, 1995; Tournebise *et al.*, 1997). In contrast, others have suggested that stathmin is required for mitotic progression (Rana *et al.*, 2008). Because our data pointed to stathmin functioning to regulate interphase MT nucleation, rather than MT plus-end dynamics, we used two measures to examine possible stathmin function in mitosis. First, we compared the number of astral MTs in stathmin $^{+/+}$ and stathmin $^{-/-}$ MEFs. As shown in Figure 5A, stathmin genotype did not impact the number of astral MTs. Second, we measured MT nucleation rate in LLC PK cells at metaphase of mitosis. As shown in Figure 5B, MT nucleation at metaphase was insensitive to moderate expression of stathmin-CFP. Our data are consis-

Figure 5. Stathmin does not regulate MT nucleation during metaphase. (A) The number of astral MTs, measured by counting EB1 marked MT tips, was similar for metaphase spindles from stathmin^{+/+} and stathmin^{-/-} MEFs. Data shown are means $\pm$ SD for 13–18 spindle poles. Images to the right are maximum intensity projections from image stacks used to count EB1 comets. EB1 (green), γ -tubulin (red) and DNA (blue) are shown. (B) MT nucleation rate was measured in LLCPC-EB1-GFP cells by counting the number of EB1-GFP comets emerging from the centrosome over time. Data shown are means $\pm$ SD from seven to 15 cells. Example of EB1-GFP image from a mitotic cell expressing stathmin-CFP is shown to the right. Nucleation rate was measured for the astral side of the centrosome, as outlined by the white half-circle. Bar, 5 μ m.

tent with previous results showing that stathmin is inactivated during mitosis (Larsson *et al.*, 1995; Tournebize *et al.*, 1997).

DISCUSSION

Stathmin Regulates Tubulin Dimer/Polymer Partitioning and MT Nucleation from Centrosomes

Manipulating stathmin expression level in noncancerous cells, either by isolation of MEFs differing in stathmin genotype or by transient overexpression of stathmin in LLCPC cells, resulted in changes in tubulin partitioning between dimer and polymer pools. Several different assays were used to measure MT polymer, including measurement of MT staining intensity, Western blot of soluble and cytoskeletal fractions, and counting both the number of MTs within 5 μ m of the cell periphery and the total number of growing MTs (EB1-GFP comets) per cell. These data demonstrate that lowering stathmin increases MT polymer, whereas raising stathmin decreases MT polymer, consistent with previous results in leukemia-derived cells (Holmfeldt *et al.*, 2007; Sellin *et al.*, 2008), newt lung epithelial cells (Howell *et al.*, 1999a), and PtK1 cells (Wittmann *et al.*, 2004). Given the low concentration of stathmin relative to tubulin in MEFs (Figure 1) and LLCPC cells (Supplemental Figure 1), it is unlikely

that tubulin-sequestering activity plays a major role in regulating tubulin dimer/polymer partitioning.

Surprisingly, the changes in MT dimer/polymer partitioning were not accompanied by large changes in MT dynamics at the cell periphery. We found that MTs remained dynamic in both MEFs lacking stathmin and in LLCPC cells overexpressing stathmin. MTs at the cell periphery grew and shortened at similar rates regardless of stathmin level, although these rates were reduced slightly in cells with either decreased or increased stathmin level (Tables 1 and 2). In general, we observed relatively small changes in MT dynamics, as measured by MT dynamicity (the total net gain and loss of tubulin dimers over time) and drift velocity (the net gain or loss of dimers over time), for cells differing in stathmin level.

It is surprising that stathmin level had relatively mild effects on MT dynamics, given its major effect on MT catastrophes and lengths in *Xenopus* egg extracts (Tournebize *et al.*, 1997) and on catastrophe frequency in newt lung epithelial cells (Howell *et al.*, 1999a). In contrast, our results agree with recent results from leukemia-derived cell lines, in which stathmin depletion did not have a detectable effect on MT sensitivity to depolymerization by nocodazole (Sellin *et al.*, 2008). The different effects of stathmin on the MT system in *Xenopus* extracts (Tournebize *et al.*, 1997) or in several cell types (Sellin *et al.*, 2008; this study) may reflect different responses of the MT system in unbounded (extract) or bounded (cells) states, in which boundary effects from the cell's plasma membrane impact dynamics (Gregorette *et al.*, 2006). It is not yet clear why stathmin regulated catastrophes in newt lung cells but had no impact on catastrophes in the two cell types studied here.

In most of our experiments, MT dynamics was measured several days or longer after stathmin level manipulation (transient overexpression or gene knockout), providing cells time to adapt and reach a new steady state. In all cases, we examined the MTs that remained at the cell periphery hours to days after manipulating stathmin level and not immediately upon a change in stathmin concentration. It is possible that up-regulation of stathmin initially increased MT catastrophes but that the system adapted to return the catastrophe rate to its baseline rate, for those MTs that remain. Such an adaptation would have to occur in <2 h, based on data from cells injected with stathmin-FLAG. We cannot formally rule out such an adaptation of the MT system but should such adaptation occur, it is not sufficient to return the MT polymer to its original level, based on MT density at the cell periphery or the number of growing MT ends per cell (Figure 4).

How then does stathmin regulate tubulin partitioning between soluble and polymer pools without major changes to MT dynamics at the cell periphery? We suggest that stathmin-dependent regulation of new MT formation at the centrosome is the critical function regulating tubulin partitioning. Nucleation rate was increased in stathmin^{-/-} MEFs (Figure 3A) and reduced in LLCPC-EB1-GFP cells overexpressing stathmin-CFP (Figure 3B). By regulating MT nucleation, stathmin contributes to setting the number of MTs per cell. We suggest that stathmin shifts tubulin dimer/polymer partitioning through regulation of MT number, rather than through regulation of MT dynamics at the cell periphery. These data support a model originally proposed by Mitchison and Kirschner (1987), demonstrating computationally that changes in the number of nucleation sites can regulate tubulin dimer/polymer partitioning (Mitchison and Kirschner, 1987).

Although our results point to a role for stathmin in regulating MT nucleation, it is not clear where this regulation occurs. Our nucleation assay uses EB1-GFP to detect new MTs as they emerge from the centrosome; therefore, we detect either nucleation or a step shortly thereafter. It is possible that nascent MTs are more sensitive to stathmin level than those MTs that reach the cell periphery, possibly because nascent MTs have not yet accumulated sufficient stabilizing MAPs to sustain growth. Alternatively, the nucleation step(s) may be more sensitive to stathmin level than is continued plus-end polymerization. In support of this idea, stathmin regulates the number of MTs nucleated from flagellar axoneme fragments *in vitro* in the absence of MAPs (Howell *et al.*, 1999b).

MT Growth Rate Seems Independent of Tubulin Dimer Concentration

Stathmin binds two tubulin dimers and can sequester those dimers to prevent their polymerization (Steinmetz, 2007); yet, we find that increased or decreased stathmin level did not significantly impact MT growth rate (Tables 1 and 2). We also estimate that stathmin is present at low concentrations relative to tubulin in both MEFs (described above) and LLCs (Supplemental Figure 1). These data indicate that stathmin does not function as a sequestering protein in noncancerous cells, consistent with our previous observations in newt lung cells (Howell *et al.*, 1999a). Surprisingly, we also found that the total tubulin pool was equal in all MEF cell lines, whereas elimination of both copies of the stathmin gene shifted most tubulin into polymer and drastically reduced the amount of unpolymerized dimers (Figure 2, C and D). Given the low concentration of tubulin dimers in stathmin^{-/-} MEFs, it is surprising that MT growth rate was only slightly slower in these cells (Table 1). These data indicate that, in cells, MT growth rate shows little variation across a surprisingly wide range of tubulin dimer concentrations, as suggested several years ago by experiments in *Xenopus* extracts and cytoplasts (Parsons and Salmon, 1997; Rodionov *et al.*, 1999).

Stathmin Regulation of the MT System

Stathmin regulates the MT system at several levels, including dimer/polymer partitioning and in some cells, tubulin expression (Fletcher and Rorth, 2007; Sellin *et al.*, 2008). For example, in Jurkat cells, stathmin depletion reduces both total tubulin protein and mRNA levels, yet at the same time shifts more tubulins into polymer (Sellin *et al.*, 2008). In *Drosophila* embryos, stathmin also regulates the total tubulin pool (Fletcher and Rorth, 2007). In MEFs, we see approximately equal total tubulin expression, but shifts in dimer/polymer partitioning, similar to previous reports for K562 cells (Holmfeldt *et al.*, 2007; Sellin *et al.*, 2008).

Stathmin is phosphorylated by a number of kinases, which reduces stathmin's MT destabilizing activity (reviewed in Cassimeris, 2002). It is likely that phosphorylation also turns off stathmin's ability to regulate MT nucleation, suggesting that MT number can be modified rapidly in response to various signal cascades. It is interesting to note that stathmin is phosphorylated as cells enter mitosis (Larsson *et al.*, 1995), at a time when nucleation of MTs increases dramatically (Kuriyama and Borisy, 1981; Piehl *et al.*, 2004). It is possible that stathmin inactivation contributes to the greater MT nucleation during mitosis, although this has not been tested directly. Here, we show that stathmin expression level did not affect either metaphase MT nucleation rate or the number of astral MTs, supporting the idea that stathmin is inactivated during mitosis. Further experiments are

needed to understand how stathmin regulates MT nucleation and the impact of this regulation on the MT system, cell cycle progression, and other stathmin-dependent processes.

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