

# CaMKII $\gamma$ -mediated inactivation of the Kin I kinesin MCAK is essential for bipolar spindle formation

Per Holmfeldt<sup>1</sup>, Xin Zhang<sup>2</sup>,  
Sonja Stenmark<sup>1</sup>, Claire E Walczak<sup>3</sup>  
and Martin Gullberg<sup>1,\*</sup>

<sup>1</sup>Department of Molecular Biology, Umeå University, Umeå, Sweden,  
<sup>2</sup>Department of Biology, Indiana University, Bloomington, IN, USA and  
<sup>3</sup>Departments of Biochemistry and Molecular Biology,  
Indiana University, Bloomington, IN, USA

**MCAK, a member of the kinesin-13 family, is a microtubule (MT) depolymerase that is necessary to ensure proper kinetochore MT attachment during spindle formation. Regulation of MCAK activity and localization is controlled in part by Aurora B kinase at the centromere. Here we analyzed human cells depleted of the ubiquitous Ca<sup>2+</sup>/calmodulin-dependent protein kinase II $\gamma$  isoform (CaMKII $\gamma$ ) by RNA interference and found that CaMKII $\gamma$  was necessary to suppress MCAK depolymerase activity *in vivo*. A functional overlap with TOGp, a MT regulator known to counteract MCAK, was suggested by similar CaMKII $\gamma$ - and TOGp-depletion phenotypes, namely disorganized multipolar spindles. A replicating vector system, which permits inducible overexpression in cells that simultaneously synthesize interfering short hairpin RNAs, was used to dissect the functional interplay between CaMKII $\gamma$ , TOGp, and MCAK. Our results revealed two distinct but functionally overlapping mechanisms for negative regulation of the cytosolic/centrosomal pool of MCAK. These two mechanisms, involving CaMKII $\gamma$  and TOGp, respectively, are both essential for spindle bipolarity in a normal physiological context, but not in MCAK-depleted cells.**

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## Introduction

The bipolar mitotic spindle segregates duplicated chromosomes during cell division. Generation of a functional spindle requires both the inherent dynamic instability of microtubules (MTs), which are polar polymers comprised of  $\alpha/\beta$  tubulin heterodimers, and regulatory proteins that alter MT dynamics (reviewed in Desai and Mitchison, 1997). Spindle MTs attach to sister chromatids at kinetochores, which are protein complexes that assemble onto centromeres (reviewed in Biggins and Walczak, 2003). Chromosome movement is

powered by a combination of motor proteins at the kinetochore and balanced MT dynamics in the spindle. Kinetochores lacking attached MTs also serve as the signal generators for the mitotic checkpoint, which arrests mitosis until all kinetochores are correctly attached. Defects in the mitotic checkpoint results in aneuploidy, which is one of the hallmarks for cancer cells.

Entry into mitosis triggers global changes in MT dynamics including an increased catastrophe frequency, that is, the transition from MT growth to shrinkage (reviewed in Kline-Smith and Walczak, 2004). In *Xenopus* egg extracts, the kinesin-13 family member MCAK mediates increased catastrophe frequency at the onset of mitosis (Tournebize *et al*, 2000; Lawrence *et al*, 2004). The mechanism of MCAK-mediated catastrophe promotion involves binding to both ends of the MT polymer and catalytically depolymerizing protofilament ends (Desai *et al*, 1999; Hunter *et al*, 2003). In *Xenopus* egg extracts (Tournebize *et al*, 2000) and in an *in vitro* MT assembly system consisting of purified components (Kinoshita *et al*, 2001), the potent catastrophe-promoting activity of MCAK is counteracted by the XMAP215 protein.

Human cells differ from *Xenopus* egg extracts in that the counteractive activities of MCAK and the human homolog of XMAP215, TOGp, are not clearly manifested at the level of stability of MT plus ends. Instead, the phenotype of cells depleted of TOGp by RNA interference has highlighted an essential function of TOGp at the centrosome (Gergely *et al*, 2003). Bipolar spindles can form in cells depleted of either MCAK alone or doubly depleted of MCAK and TOGp, but not in cells depleted of TOGp alone (Cassimeris and Morabito, 2004; Holmfeldt *et al*, 2004). Given that endogenous MCAK is required for manifestation of the characteristic TOGp-depletion phenotype of disorganized multipolar spindles, we proposed that centrosomal TOGp has an essential role in protecting spindle MTs from MCAK activity at the centrosome (Holmfeldt *et al*, 2004).

For accurate chromosome segregation during anaphase, it is essential that each kinetochore of a pair of sister chromatids attaches to spindle MTs from opposing poles, such that all chromosomes are bioriented. MCAK is necessary for proper chromosome segregation (Maney *et al*, 1998) by mediating proper MT attachments during congression (Walczak *et al*, 2002; Kline-Smith *et al*, 2004). Recent studies have shown that the Aurora B kinase and the inner centromere Kin I stimulator ICIS function with MCAK at the centromere to regulate proper MT attachment (Ohi *et al*, 2003, 2004; Andrews *et al*, 2004; Lan *et al*, 2004). The emerging model implies that Aurora B and ICIS regulate MCAK activity at specific regions within the centromere, such that the depolymerizing activity of MCAK is directed towards incorrectly orientated kinetochore MTs.

Spatial and temporal regulation of MCAK by the Aurora B kinase involves phosphorylation on multiple Ser residues, which suppresses and thereby controls the potent MT-destabilizing activity of MCAK at the centromere (Andrews *et al*, 2004; Lan *et al*, 2004; Ohi *et al*, 2004). However, MCAK is also

\*Corresponding author. Department of Molecular Biology, Umeå University, S-901 87 Umeå, Sweden. Tel.: +46 90 785 67 03; Fax: +46 90 77 14 20; E-mail: martin.gullberg@molbiol.umu.se

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abundant in the cytosol and at the centrosome, and it is unclear how its activity is controlled at these locations. In contrast to what could be expected from the original studies of *Xenopus* egg extracts (Walczak *et al*, 1996; Tournebize *et al*, 2000), it was recently shown that MCAK depletion in human cells by RNA interference has only a minor effect on the level of spindle MT polymer content and no effect on the density of the interphase MT array (Cassimeris and Morabito, 2004; Holmfeldt *et al*, 2004). This surprisingly modest depletion phenotype suggests that cytosolic/centrosomally located MCAK is also subject to negative regulation, but the mechanism is unknown.

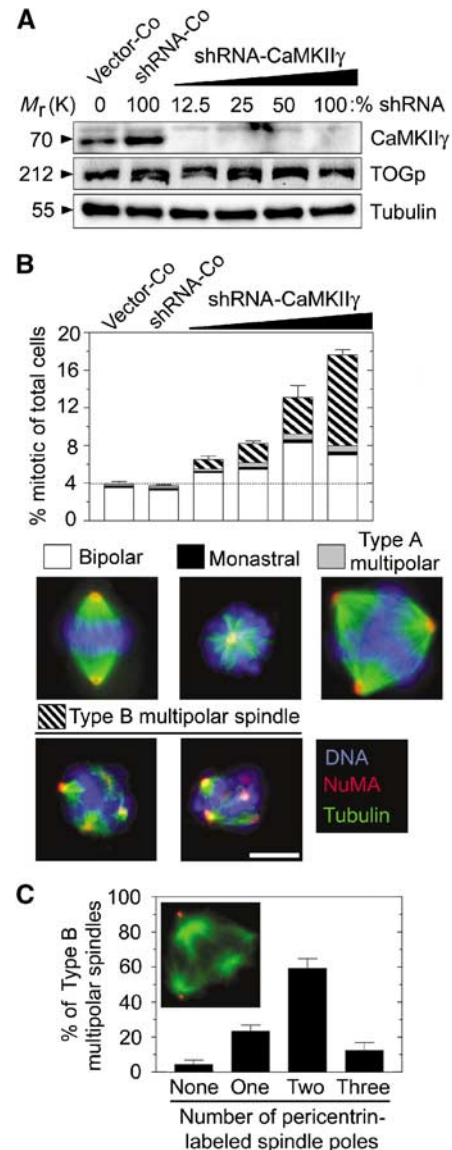
Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaMKII) is a multifunctional enzyme that has been implicated in a multitude of Ca<sup>2+</sup>-regulated biological processes, including synaptic transmission, gene transcription, and growth control (reviewed in Hudmon and Schulman, 2002). There are four isoforms of CaMKII encoded by separate genes; the  $\gamma$  and  $\delta$  isoforms are ubiquitously expressed, while the  $\alpha$  and  $\beta$  isoforms are neural specific. Here we have investigated functions of CaMKII $\gamma$  in human cell lines, either by depleting the  $\gamma$  isoform by RNA interference or by overexpressing a constitutively active derivative. These studies revealed an essential function of CaMKII $\gamma$  as a negative regulator of MCAK that is required for suppression of cytosolic/centrosomal MCAK activity during spindle assembly. CaMKII $\gamma$  depletion resulted in the same type of disorganized multipolar spindles that were previously observed in TOGp-depleted cells. By examining the functional interplay between CaMKII $\gamma$ , TOGp, and MCAK, we propose a model in which this interplay is essential for protection of MTs from uncontrolled MCAK activity at the centrosome.

## Results

### CaMKII $\gamma$ is essential for bipolar spindle formation

To explore a potential cell cycle regulatory role of the ubiquitously expressed CaMKII $\gamma$  isoform, CaMKII $\gamma$  levels were reduced in human K562 leukemia cells by RNA interference by using a short hairpin RNA (shRNA) expression vector. Our approach relies on an EBV-based replicating vector system that confers hygromycin resistance and thus allows selection of cells within 3 days, all of which express the transfected shRNA (Holmfeldt *et al*, 2004). Graded shRNA expression levels were achieved by premixing the specific shRNA derivative at different ratios with a control vector. As these vectors have a stringent replication control, the relative proportion of transfected DNA is maintained (Melander Gradin *et al*, 1997). Transfection of a shuttle vector directing expression of CaMKII $\gamma$ -specific shRNA (shRNA-CaMKII $\gamma$ )-depleted CaMKII $\gamma$ , while TOGp and tubulin levels were unaltered (Figure 1A). Quantification of immunoblots by serial dilutions of cell lysates revealed more than 90% specific depletion at all shRNA-CaMKII $\gamma$  dilutions. It should be noted that >95% reduction of CaMKII $\gamma$  was below the limit of detection.

Analysis of CaMKII $\gamma$ -depleted cells revealed no detectable phenotype on the level of MT polymer in interphase cells, suggesting that endogenous CaMKII $\gamma$  is not important for regulating the density of the interphase MT array (data not shown). However, whereas cells harboring a control vector (Vector-Co) or cells expressing a control shRNA with a



**Figure 1** Phenotype of K562 cells expressing CaMKII $\gamma$ -specific interfering shRNA. Cells were transfected with a total of 18  $\mu$ g DNA comprising the indicated percentage of shRNA-scrambled or shRNA-CaMKII $\gamma$  made up of Vector-Co. Cells were counter-selected for 5 days with hygromycin to kill off nontransfected cells. (A) Immunoblots of total cellular lysates using the indicated antibody for detection. (B) Cells were triple-stained for DNA, MTs, and the MT minus-end marker NUMA. Representative examples of each spindle type are shown below the histogram. Spindles of mitotic cells were categorized into four groups, namely, bipolar, monastral, and multipolar types A and B, as indicated by the subdivision of bars. The data in this and subsequent figures are presented as percentage of total cells and represent the means of duplicate determinations of coded samples ( $n = 300$  mitotic cells). The most notable difference between type A and type B multipolar spindles is that the chromosomes of type B spindles are completely disorganized and appear aggregated (two representative examples shown). Bar: 10  $\mu$ m. (C) Cells were transfected with 100% shRNA-CaMKII $\gamma$  and counter-selected for 5 days with hygromycin. The histogram depicts the distribution of the number of spindle poles in individual cells with type B multipolar spindles, as determined by pericentriole-labeled dots positioned at the spindle pole ( $n = 40$ ). The data represent the means of duplicate determinations. A representative epifluorescence image of a type B multipolar spindle of a shRNA-CaMKII $\gamma$ -expressing cell stained with anti- $\alpha$ -tubulin (green) and anti-pericentrin (red) is also shown. The data in this and subsequent figures are representative of at least three independent transfection experiments.

scrambled sequence (shRNA-Co) were unaffected, analysis of cell cycle profiles after 5 days of CaMKII $\gamma$  depletion revealed an increased fraction of cells with G2/M DNA content (data not shown) and accumulation of mitotic cells with multipolar spindles (see subdivision of bars in Figure 1B). This phenotype became increasingly pronounced at increasing shRNA-CaMKII $\gamma$  concentrations. The multipolar spindles observed could be divided into two categories: (1) multipolar spindles with chromosomes that appear well organized between three or more spindle poles (type A), which are also observed among Vector-Co cells, and (2) disorganized multipolar spindles with multiple MT asters of various sizes and with disorganized chromosomes aggregated around each aster (type B) (see images in Figure 1B). The organization of MTs at the minus end in the type B multipolar spindles was not defective, as evidenced by NUMA staining. We found that the type B multipolar spindles were not observed among control cells and are therefore a hallmark of CaMKII $\gamma$ -depleted cells.

The shRNA-CaMKII $\gamma$  derivative used here depletes its target more efficiently than the shRNA-CaMKII $\gamma$  derivative that was used as a negative control in a previous study (Holmfeldt *et al*, 2004). This observation, as well as a comparison of immunoblot analysis (Figure 1A) and the finding of accumulation of defective mitotic cells (Figure 1B), emphasizes the requirement of efficiently depleting CaMKII $\gamma$  in order to detect a phenotype. To ascertain that the phenotype observed was specific for CaMKII $\gamma$  depletion, we also analyzed the effect of an independent CaMKII $\gamma$ -specific shRNA derivative and the CaMKII inhibitory drug KN93. In both cases, type B multipolar spindles were generated (data not shown). This spindle phenotype was also observed as a major phenotype in the Jurkat T-cell leukemia and the DG 75 Burkitt lymphoma cell lines expressing either of two distinct shRNA-CaMKII $\gamma$  derivatives (data not shown). Thus, the ubiquitously expressed CaMKII $\gamma$  appears to be necessary for efficient bipolar spindle formation in human cells.

To look more closely at the organization of poles in CaMKII $\gamma$ -depleted cells, the type B multipolar spindles of CaMKII $\gamma$ -depleted cells were further characterized by dual labeling of tubulin and the centrosomal protein pericentrin. We found that the majority of these abnormal spindles in shRNA-CaMKII $\gamma$ -expressing cells contained centrosomes at only two of the multiple spindle poles (Figure 1C), which shows that type B multipolar spindles are not caused by nucleation from multiple centrosomes.

### **The CaMKII $\gamma$ -depletion phenotype is dependent on MCAK**

It is notable that the type B multipolar spindles observed among CaMKII $\gamma$ -depleted cells appear indistinguishable from the multipolar spindles observed after depletion of TOGp (Gergely *et al*, 2003). It has previously been shown that the type B multipolar spindles caused by depletion of TOGp are dependent on MCAK (Cassimeris and Morabito, 2004; Holmfeldt *et al*, 2004). To determine if MCAK is also involved in generating the CaMKII $\gamma$ -depletion phenotype, cells were either singly or doubly depleted of CaMKII $\gamma$  and MCAK by expressing specific interfering shRNAs. Cell proliferation and mitotic figures were followed over 10 days and compared to cells transfected with Vector-Co or a scrambled control deri-

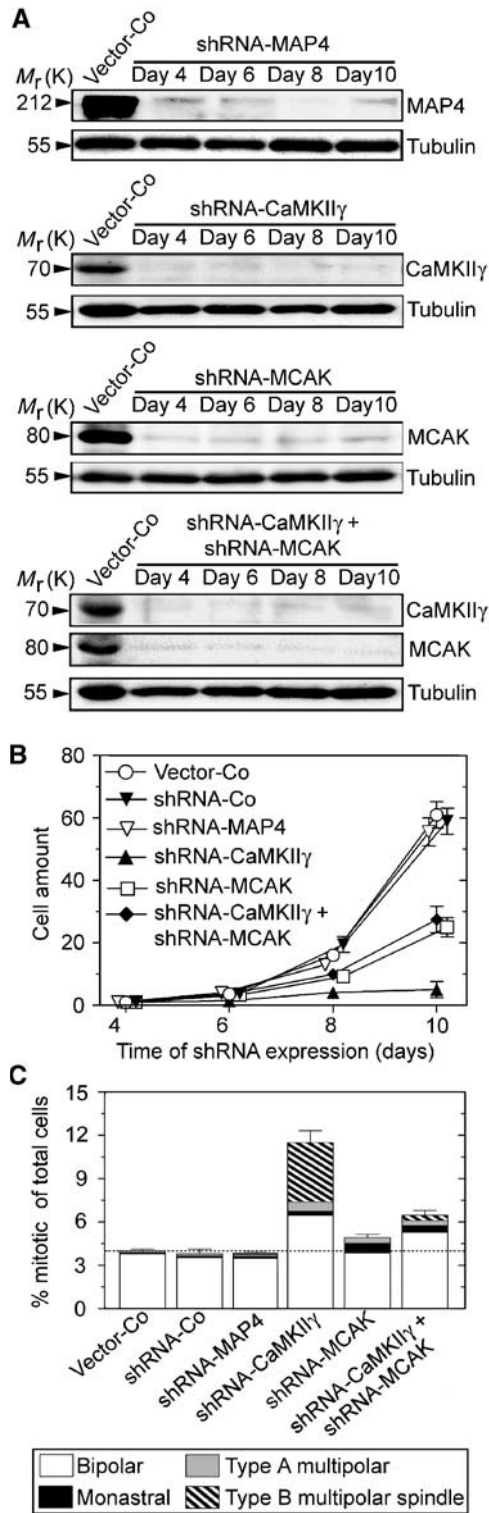
vative (shRNA-Co). As an additional control for the specificity of phenotypes, we also included shRNA-MAP4 to deplete the MT-associated MAP4 protein, which is not required for spindle assembly (Wang *et al*, 1996). It is shown in Figure 2A that the present system for RNA interference allows depletion of the specific gene product for prolonged culture periods and, as expected, depletion of MAP4 did not cause interference with cell proliferation (Figure 2B) or spindle assembly (Figure 2C). Moreover, depletion of MCAK caused a modest inhibition of cell proliferation, which is consistent with our previous report (Holmfeldt *et al*, 2004), whereas depletion of CaMKII $\gamma$  caused an almost complete growth arrest. Most importantly, simultaneous depletion of MCAK and CaMKII $\gamma$  greatly reduced the cell proliferation defect caused by depletion of CaMKII $\gamma$  alone and proliferation of doubly depleted cells was very similar to that observed after depletion of MCAK alone. These results suggest that MCAK is involved in growth inhibition of CaMKII $\gamma$ -depleted cells.

Consistent with our previous study (Holmfeldt *et al*, 2004), analysis of mitotic figures revealed a relatively mild MCAK-depletion phenotype in which there was a slight increase in the number of cells with monastral spindles (Figure 2C). We also observed the expected accumulation of mitotic cells in the CaMKII $\gamma$ -depleted population, many of which contained multipolar spindles (Figure 2C). It should be noted that the shRNA-CaMKII $\gamma$  has to be diluted with vector control DNA to allow direct comparisons with doubly depleted cells (i.e., 50% of each shRNA in the transfection mix); therefore, the mitotic CaMKII $\gamma$ -depletion phenotype is not as dramatic as for maximally depleted cells (i.e., 100% shRNA-CaMKII $\gamma$  DNA, Figure 1). Most importantly, double depletion of MCAK and CaMKII $\gamma$  significantly reduced the mitotic index and spindle abnormalities caused by depletion of CaMKII $\gamma$  alone. In fact, the defects in the doubly depleted cells appeared similar to the relatively mild mitotic phenotype of MCAK depletion alone. Consistent with the cell proliferation assays, these data show that the presence of MCAK is necessary for the mitotic phenotypes caused by depletion of CaMKII $\gamma$ , perhaps by a mechanism in which CaMKII $\gamma$  down-regulates MCAK activity.

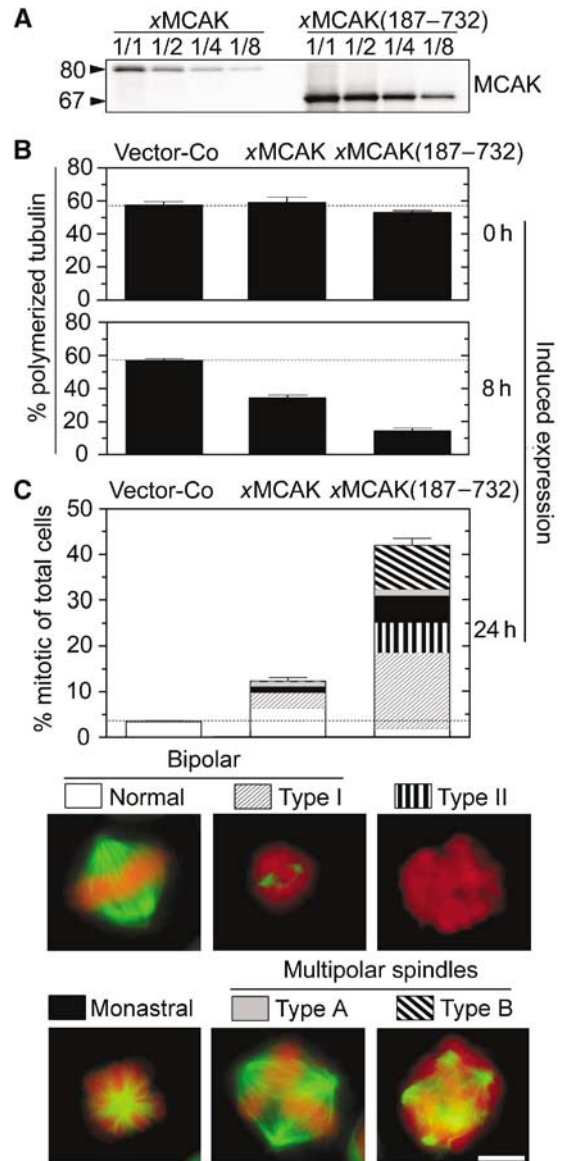
The mitotic phenotype of CaMKII $\gamma$  depletion includes not only type B multipolar spindles, but also an increased population of bipolar spindles (see subdivision of bars in Figure 2C). Classification of these bipolar spindles revealed an accumulation of prometaphase/metaphase cells, which we found in CaMKII $\gamma$ -depleted cells but not in cells depleted of MCAK or doubly depleted of CaMKII $\gamma$  and MCAK (Supplementary Figure S1). Many of the prometaphase spindles found in CaMKII $\gamma$ -depleted cell populations appeared unusually small, but these features were not distinctive enough to allow a clear-cut classification into a specific subgroup. Nevertheless, given that accumulation of prometaphase/metaphase cells in the CaMKII $\gamma$ -depleted population is dependent on MCAK, and that excessive cytosolic MCAK activity is likely to cause accumulation of prometaphase/metaphase cells, these results suggest that CaMKII $\gamma$  depletion results in uncontrolled cytosolic MCAK activity.

### **Excessive MCAK levels cause type B multipolar spindles besides other spindle abnormalities**

If uncontrolled MCAK activity is the direct cause of the type B multipolar spindle abnormality, we would expect that

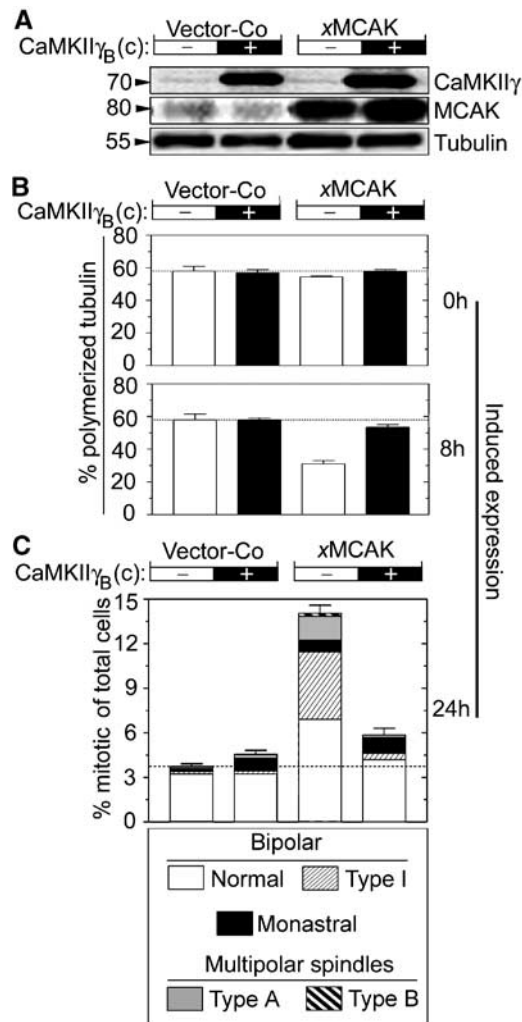


**Figure 2** The essential function of CaMKII $\gamma$  during cell division is dependent on MCAK. Cells were co-transfected, as in Figure 1, with DNA mixtures containing either 50% of each shRNA, or individually with 50% Vector-Co. Nontransfected cells were counter-selected by culturing in the presence of hygromycin, which killed off most nontransfected cells within 3 days. (A) Total cellular lysates were analyzed by immunoblotting using the indicated antibodies for detection. (B) Viable cells grown in the presence of hygromycin were determined on the days indicated. The graph represents the mean  $\pm$  s.d. of data from three independent transfection experiments, each of which was performed in duplicate. (C) Mitotic figures were categorized after 6 days of depletion, as described under Figure 1B.



**Figure 3** MT depolymerization and mitotic defects caused by high levels of xMCAK active in the cytosol and/or the centrosome. K562 cells were transfected with the pMEP derivative indicated (18  $\mu$ g) and counter-selected with hygromycin for 5 days. Cd<sup>2+</sup> was added to induce ectopic xMCAK from the hMTIIIa promoter for the indicated time period. (A) To compare expression of pMEP-xMCAK(187-732), which contains an optimized 5' untranslated sequence, and pMEP-xMCAK, serially diluted lysates from Cd<sup>2+</sup>-induced (8 h) transfected cells were analyzed by immunoblotting (1/1: 25  $\mu$ g/lane; 1/2: 12.5  $\mu$ g/lane; 1/4: 6.3  $\mu$ g/lane; 1/8: 3.1  $\mu$ g/lane). To allow comparison of full-length and N-terminal truncated xMCAK proteins, an antiserum raised against the C-terminus of xMCAK was used for detection. (B) MT content expressed as percentage polymerized tubulin after 0 and 8 h of induced expression. (C) After 24 h of induced expression, mitotic figures were categorized as described in Figure 1B. Representative examples of each spindle type found among xMCAK(187-732)-expressing cells are shown below. Characteristic features of type I bipolar spindles as compared to normal spindles include the observation that most kinetochore MTs are absent and MTs appear as two dense star-like asters. Bar: 10  $\mu$ m.

increasing the levels of cytosolic and/or centrosomal MCAK will at some point out-titrate the negative regulation by CaMKII $\gamma$ , and thereby generate type B multipolar spindles in addition to other mitotic abnormalities. To test this model,



**Figure 4** Constitutively active CaMKII $\gamma_B(c)$  suppresses the activity of ectopic xMCAK during both interphase and mitosis. K562 cells were co-transfected (18  $\mu$ g in total, DNA ratio 1:1) as indicated, with either Vector-Co or pMEP-xMCAK combined with either Vector-Co (-) or pMEP-CaMKII $\gamma_B(c)$  (+). After 5 days of counter-selection with hygromycin, Cd<sup>2+</sup> was added to induce ectopic expression from the hMTIIa promoter of the pMEP vector. (A) Immunoblots of total cellular lysates from Cd<sup>2+</sup>-induced cells (8 h) using the indicated antibody for detection. (B) MT content in interphase cells expressed as percentage of polymerized tubulin prior to (0 h) or after Cd<sup>2+</sup>-induced expression (8 h). (C) After 24 h of induced expression, mitotic cells were categorized as described in Figure 1B, into the six groups depicted in Figure 3C.

we created a pMEP-xMCAK derivative that lacks the N-terminal centromere-targeting domain and, consequently, will only act in the cytosol and at the centrosome (Walczak *et al*, 2002). In addition, to increase the expression level, the coding region of xMCAK was fused with a 5' untranslated region that directs high-level protein expression in human cells. The resulting xMCAK(187-732) derivative was expressed four- to eight-fold higher than the intact xMCAK derivative (Figure 3A). At these high levels, interphase MTs were almost completely depolymerized within 8 h (Figure 3B). Consistent with increased expression levels, cells expressing xMCAK(187-732) for 24 h showed a much higher mitotic index than cells expressing xMCAK (Figure 3C).

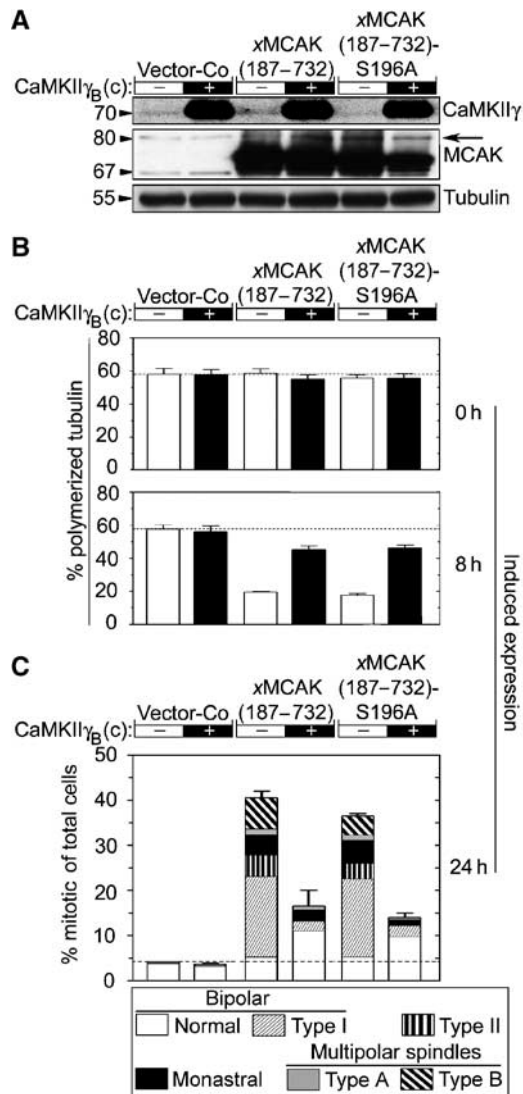
Analysis of mitotic figures revealed that essentially all xMCAK(187-732)-expressing mitotic cells had some type of distinctive abnormality, which were categorized into five distinct types (Figure 3C). We found that expression of either xMCAK derivative caused a major increase in the percentage of the previously described type I bipolar spindle (see subdivision of bars in Figure 3C), which has characteristic small MT asters that are indicative of excess catastrophe promotion (Holmfeldt *et al*, 2004). Moreover, in cells expressing high levels of xMCAK(187-732), many mitotic cells were completely devoid of detectable MTs, which indicates complete depolymerization (termed type II). Importantly, while analysis of mitotic figures revealed very few type B multipolar spindles among cells expressing the full-length xMCAK derivative, essentially all multipolar spindles in cells expressing xMCAK(187-732) were the disorganized type B, and this spindle type comprised ~20 % of the accumulated mitotic cells. Thus, MCAK has, at sufficiently high levels, the potential to generate a fraction of type B multipolar spindles. This is consistent with a model in which unregulated MCAK activity is the cause of the multipolar spindle phenotype seen when CaMKII $\gamma$  is inhibited.

#### Ectopic CaMKII $\gamma$ suppresses the MT-depolymerizing activity of ectopic xMCAK

If CaMKII $\gamma$  is responsible for downregulating MCAK activity, then we would also expect that overexpressing active CaMKII $\gamma$  would suppress the defects seen upon overexpression of xMCAK. To test this idea, we coexpressed xMCAK with a constitutively active CaMKII $\gamma_B(c)$  derivative (CaMKII $\gamma_B(c)$ ). To allow selection of cell populations that coexpress two potentially growth-inhibitory gene products, we used a previously described system for inducible coexpression of genes encoding a substrate and a cognate kinase carried on pMEP vectors under the control of the tightly regulatable hMTIIa promoter (Gradin *et al*, 1998). We found that stimulation of the hMTIIa promoter for 8 h resulted in a six- to 10-fold overexpression of CaMKII $\gamma_B(c)$  and xMCAK proteins as compared to the cognate endogenous gene products (Figure 4A).

Analysis of the total MT content in interphase cells prior to induced expression (0 h) revealed similar tubulin polymer levels in all four transfected cell populations (Figure 4B), which reflects the tight regulation from the hMTIIa promoter. Expression of CaMKII $\gamma_B(c)$  alone caused no changes in the MT polymer level after 8 h, while expression of xMCAK caused ~2-fold reduction in the MT content. We found that the MT depolymerization caused by xMCAK overexpression was efficiently suppressed by coexpressed CaMKII $\gamma_B(c)$ , which is consistent with our model wherein MCAK activity is downregulated by CaMKII $\gamma$ . To explore the functional interactions between ectopic CaMKII $\gamma_B(c)$  and xMCAK during spindle formation, the mitotic index and the appearance of mitotic cells were analyzed after 24 h of induced expression. CaMKII $\gamma_B(c)$  overexpression caused only a very subtle phenotype (Figure 4C). In contrast, high levels of xMCAK expression caused the expected accumulation of mitotic cells, which frequently displayed the bipolar type I abnormality described above (Figure 3). Most significantly, if xMCAK was coexpressed with CaMKII $\gamma_B(c)$ , the frequency of mitotic cells was close to normal, and the percentage of cells with type I abnormal spindles was reduced to control levels. These data





**Figure 5** CaMKII $\gamma_B$ (c) suppresses MCAK-dependent generation of defective spindles independently of the major negative Aurora B phosphorylation site. K562 cells were co-transfected as in Figure 4 with either Vector-Co or the indicated pMEP-xMCAK(187-732) derivative combined with either Vector-Co (-) or pMEP-CaMKII $\gamma_B$ (c) (+). After 5 days of counter-selection with hygromycin, Cd<sup>2+</sup> was added to induce ectopic expression from the hMTIIa promoter of the pMEP vector. (A) Immunoblots of total cellular lysates from Cd<sup>2+</sup>-induced cells (8h) using the indicated antibody for detection. The migration of endogenous MCAK is indicated by an arrow. (B) MT content in interphase cells expressed as percentage polymerized tubulin prior to (0h) or after Cd<sup>2+</sup>-induced expression (8h). (C) After 24h of induced expression, mitotic cells were categorized as described under Figure 1B, into the six groups depicted in Figure 3C.

show that, similar to the observed suppression of MT depolymerization by ectopic xMCAK activity in interphase cells shown in panel B, CaMKII $\gamma_B$ (c) efficiently suppresses the spindle disruptive effect of excessive xMCAK levels during mitosis. It should be noted that we aimed for an expression level of CaMKII $\gamma_B$ (c) that by itself causes a minimal phenotype, which was restricted to a limited increase in monastral spindles. Under these conditions, the observed suppression of MCAK-dependent abnormalities appears remarkably specific.

To evaluate the specificity of CaMKII $\gamma_B$ (c)-mediated suppression further, we analyzed mechanistically distinct types of MT destabilization. We found that ectopic CaMKII $\gamma_B$ (c) neither modulated the potency by which the drug nocodazole destabilizes interphase MTs (Supplementary Figure S2), nor suppressed the activity of the MT-destabilizing protein Op18/Stathmin during interphase and mitosis (Supplementary Figure S3). These results show that CaMKII $\gamma_B$ (c) does not support MT stability against any depolymerizer, and they are consistent with a model in which CaMKII $\gamma_B$ (c) suppresses MT destabilization through MCAK suppression. This implies that ectopic CaMKII $\gamma_B$ (c) should have the same effect on the MT system as MCAK depletion, which indeed appears to be the case since either ectopic CaMKII $\gamma_B$ (c) or MCAK depletion caused a small accumulation of monastral spindles without having an effect on the MT density of interphase cells (Figure 4; Holmfeldt *et al*, 2004).

It was shown previously that MCAK is phosphorylated by Aurora B, which inhibits the depolymerization activity of MCAK (Andrews *et al*, 2004; Lan *et al*, 2004; Ohi *et al*, 2004). To determine if the previously identified Aurora B kinase phosphorylation sites were the regulatory sites used by CaMKII, CaMKII $\gamma_B$ (c) was coexpressed with an N-terminal truncated xMCAK(187-732) derivative with or without Ala substitutions at Ser-196, which is the only major negative regulatory Aurora B phosphorylation site that remains after N-terminal truncation (Lan *et al*, 2004). We found that CaMKII $\gamma_B$ (c) suppressed the potent destabilization of the interphase MT array caused by either xMCAK(187-732) or xMCAK(187-732)-S196A overexpression (Figure 5B). Moreover, analysis of mitotic figures revealed that CaMKII $\gamma_B$ (c) also mediated suppression of the potent mitotic block and accumulation of all characteristic spindle aberrancies (Figure 5C). Consistent with our data on interphase cells, suppression of overexpressed MCAK was unaltered by Ala substitution at Ser-196. These data show that suppression of MCAK activity by CaMKII $\gamma$  does not require phosphorylation of any of the previously identified Aurora B sites.

To determine whether the effects of CaMKII suppression of MCAK activity were direct, we pre-phosphorylated xMCAK *in vitro* with recombinant CaMKII and then assayed MT depolymerization activity. We found that MCAK was readily phosphorylated by CaMKII, but phosphorylation by this kinase did not inhibit the MT depolymerization activity of MCAK under any of the conditions tested (Supplementary Figure S4). Thus, it seems that CaMKII $\gamma_B$ (c) suppresses xMCAK activity in intact cells by an indirect mechanism. As removal of the Aurora B phosphorylation sites on MCAK also did not inhibit the functional relationship between MCAK and CaMKII, our data suggest that the intermediate player is not Aurora B kinase.

### Functional interplay of CaMKII $\gamma$ , MCAK, and TOGp during spindle formation

XMAP215, which is the *Xenopus* ortholog of TOGp, and xMCAK act in an antagonistic manner during spindle assembly in *Xenopus* egg extracts and in an MT assembly system consisting of purified components (Tournebize *et al*, 2000; Kinoshita *et al*, 2001). It follows that the observed phenotypes of CaMKII $\gamma$  depletion/overexpression can in principle be explained by differential modulation of the activity of either of these two MT regulators. It was therefore important to

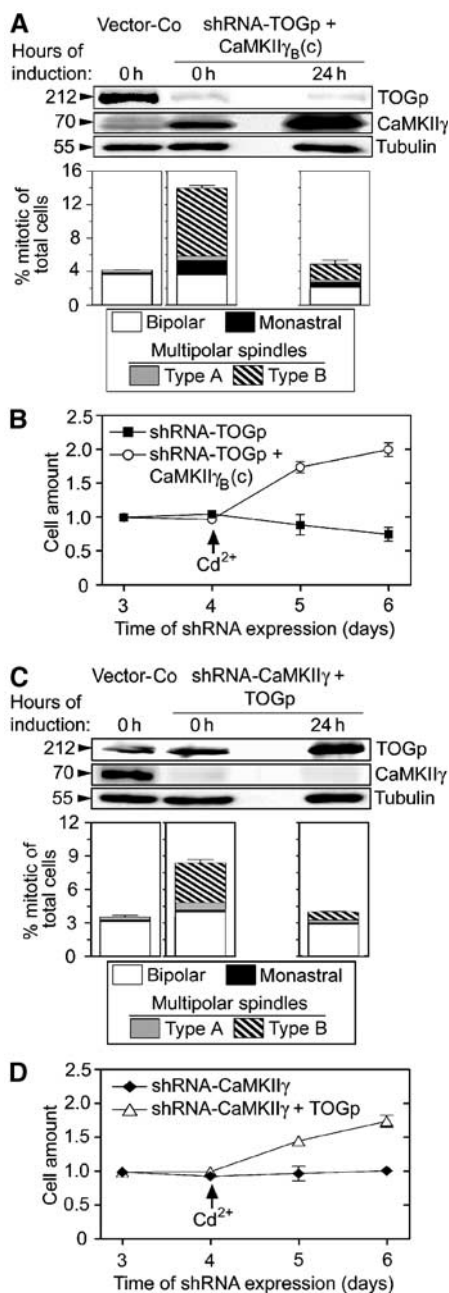
establish whether constitutively active CaMKII $\gamma$  suppresses the type B multipolar spindle phenotype in TOGp-depleted cells, as would be predicted by suppression of cytosolic/centrosomal MCAK activity. Replicating vectors that direct constitutive synthesis of shRNA-TOGp and inducible expression of CaMKII $\gamma_B(c)$  were co-transfected, and a TOGp-depleted cell line was generated by counter-selection for 4 days with hygromycin. This system allowed regulated ectopic CaMKII $\gamma_B(c)$  expression in a TOGp-depleted cell line (Figure 6A). Prior to induced CaMKII $\gamma_B(c)$  expression (0 h), the mitotic index was high and type B multipolar spindles were the most common mitotic abnormality among TOGp-depleted cells. However, 24 h of induced CaMKII $\gamma_B(c)$  expression decreased the mitotic index, and the total frequency of type B spindles was suppressed  $\sim$ 5-fold (Figure 6A). In addition, CaMKII $\gamma_B(c)$  expression not only suppressed gen-

eration of type B spindles but also facilitated cell division in TOGp-depleted cells (Figure 6B). Given the previously demonstrated requirement of MCAK for manifestation of the type B multipolar phenotype in TOGp-depleted cells (Holmfeldt *et al*, 2004), the present data are consistent with a model in which CaMKII $\gamma$  suppresses the activity of centrosomal and/or cytosolic MCAK as depicted in Figure 7.

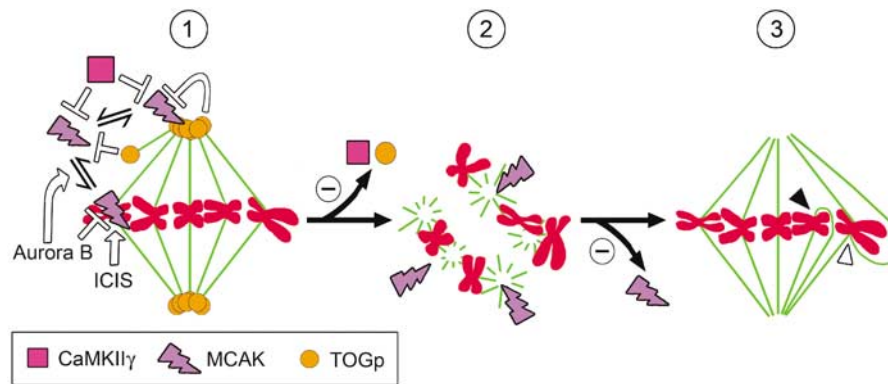
We have previously proposed that TOGp is essential at the centrosome for protection of the minus end of spindle MTs from MCAK (see Figure 7; Holmfeldt *et al*, 2004). The present study suggests an essential role of CaMKII $\gamma$  to control MCAK activity at the centrosome and that failure to do so results in the same apparent phenotype as TOGp depletion. This implies that the endogenous level of TOGp is not sufficient for protection of MT minus ends from uncontrolled MCAK activity under conditions of CaMKII $\gamma$  depletion. It was therefore of interest to evaluate if induced TOGp overexpression counteracts the apparent uncontrolled MCAK activity that results from CaMKII $\gamma$  depletion and thereby suppresses the generation of type B multipolar spindles in CaMKII $\gamma$ -depleted cells. By using the same principle experimental design as in Figure 6A and B, TOGp overexpression was induced in cells that had expressed shRNA-CaMKII $\gamma$  for 4 days (Figure 6C). We found that overexpression of TOGp in CaMKII $\gamma$ -depleted cells decreased the frequency of type B multipolar spindles (Figure 6C) and facilitated cell division (Figure 6D). Thus, given the assumption that uncontrolled MCAK activity is the cause of type B multipolar spindles in CaMKII $\gamma$ -depleted cells, these data show that increased TOGp expression provides increased protection against uncontrolled spindle disruptive MCAK activity. This is consistent with the counteractive activities of TOGp and MCAK at the centrosome as depicted in Figure 7.

## Discussion

We found that the ubiquitous  $\gamma$  isoform of CaMKII was essential for bipolar spindle formation in all three human



**Figure 6** A reciprocal functional link between CaMKII $\gamma$  and TOGp as revealed by inducible ectopic expression in cells depleted of specific products via the indicated shRNAs. Cells were transfected either with pMEP vector alone or co-transfected with a mixture of replicating shuttle vectors that direct constitutive synthesis of shRNA-TOGp and inducible expression of CaMKII $\gamma_B(c)$  (18  $\mu$ g total DNA comprising 20% shRNA-TOG and 80% pMEP-CaMKII $\gamma_B(c)$ ). After 4 days of culture, Cd $^{2+}$  was added for 24 h to specifically induce ectopic CaMKII $\gamma_B(c)$  from the hMTIIa promoter of the pMEP vector. (A) Upper panels show immunoblots of total cellular lysates using the indicated antibody for detection, prior to or after induced CaMKII $\gamma_B(c)$  expression. Lower panels show the distribution of the indicated types of mitotic figures, which were categorized as described under Figure 1B after 0 and 24 h of induced CaMKII $\gamma_B(c)$  expression. (B) The transfected cells described in panel A were counted on the indicated day after transfection. Cells were cultured for the first 4 days in media designed to suppress the hMTIIa promoter. As indicated by an arrow, the media was changed to Cd $^{2+}$ -containing media to induce a high level of CaMKII $\gamma_B(c)$  expression at day 4. The data represent the means of duplicate determinations. (C) Cells were transfected, cultured and Cd $^{2+}$ -induced as in panel A after transfection with the indicated mixture of replicating shuttle vectors (18  $\mu$ g total DNA comprising 50% shRNA-CaMKII $\gamma$  and 50% pMEP-TOGp). Analyses of protein expression and mitotic figures was performed as in panel A. (D) Transfected cells described in panel C were cultured in the absence and presence of Cd $^{2+}$ , as described under panel B.



**Figure 7** Model for MCAK involvement in the generation of type B multipolar spindles. (1) Depiction of negative regulation of MCAK by TOGp and CaMKII $\gamma$  at both free MT plus ends and at the centrosome. The previously described regulation of centromere/kinetochore-associated MCAK is also depicted, that is, centromere targeting and phosphorylation inactivation by Aurora B and stimulation of MCAK activity by ICIS. (2) Depletion of either CaMKII $\gamma$  or TOGp, or excessive levels of ectopic cytosolic/centrosomal MCAK, generates type B multipolar spindles. It is assumed that uncontrolled MCAK activity generates this defective spindle type by acting on spindle MT minus ends. (3) Co-depletion of MCAK suppresses the type B multipolar spindle phenotype, and most spindles in co-depleted cells become bipolar and at least partially functional. The appearance of multipolar spindles can also be suppressed by MCAK inhibition through ectopic CaMKII $\gamma_{B(c)}$ , as demonstrated in either TOGp-depleted cells or cells expressing excessive levels of xMCAK(187–732). It follows that CaMKII $\gamma$  and TOGp are only strictly required for spindle bipolarity, in as much as they are both essential to control cytoplasmic/centrosomal MCAK activity. As indicated in the model, however, many of the bipolar spindles formed in cells lacking active MCAK at the kinetochores can be defective with respect to kinetochore attachment, as shown by previous reports. This may lead to syntelic (closed arrowheads) or merotelic (open arrowheads) attachments.

cell lines of hematopoietic origin analyzed. It is presently unknown if the other CaMKII family members share this essential function in some non-neural or neural cell types. It is notable that even low CaMKII $\gamma$  levels are sufficient to maintain the essential mitotic function of this kinase and a substantial phenotype requires more than 90% depletion (Figure 1). Based on the effect of inhibitory peptides and drugs, in many reports CaMKII has been implicated as the effector of Ca<sup>2+</sup>-mediated signaling during mitosis (reviewed in Hudmon and Schulman, 2002).

The present study reveals important functional links between CaMKII $\gamma$ , TOGp, and MCAK. By combining shRNA-mediated depletion and inducible ectopic expression, we have recently provided evidence for three distinct levels of interdependencies between TOGp and MCAK in human cells (Holmfeldt *et al*, 2004). The first level involves a role of TOGp in protecting spindle MTs from cytosolic/centrosomal MCAK activity, which prevents formation of disorganized multipolar spindles. The second level involves TOGp-dependent counteraction of excessive MCAK activity during mitosis, which recapitulates the previously established plus-end specific counteractive activities in *Xenopus* egg extracts and *in vitro*. The third level involves an unexpected destabilization of the interphase MT array by overexpressed TOGp, which was dependent on endogenous MCAK.

Overexpression of CaMKII $\gamma$  alone does not alter the MT content in interphase cells, which illustrates a distinction between the overexpression phenotypes of CaMKII $\gamma$  and TOGp. However, there are striking similarities in the depletion and overexpression phenotypes of CaMKII $\gamma$  and TOGp in mitotic cells. First, depletion of CaMKII $\gamma$  as well as TOGp results in MCAK-dependent generation of type B multipolar spindles. Second, overexpression of CaMKII $\gamma$  as well as TOGp suppresses the spindle disrupting effect of excessive MCAK levels (Figures 2, 4, 5, and 6; Holmfeldt *et al*, 2004). The type B multipolar spindles observed in TOGp- and CaMKII $\gamma$ -de-

pleted cells appear closely related by morphological criteria. Direct evidence for a close relationship was provided by our finding that ectopic expression of both TOGp and CaMKII $\gamma$  reciprocally suppresses the generation of type B multipolar spindles and facilitates cell division in cells depleted of CaMKII $\gamma$  or TOGp (Figure 6). Another level of similarity between CaMKII $\gamma$  and TOGp is that high (>10-fold) overexpression of these two proteins alone does not cause a substantial mitotic phenotype, while overexpression of either is sufficient for suppression of the spindle disrupting activity of overexpressed xMCAK (Figures 4 and 5; Holmfeldt *et al*, 2004). Thus, bipolar spindle assembly in human cells is not critically dependent on excessive TOGp or CaMKII $\gamma$  levels being balanced by MCAK. In the case of TOGp, this finding contrasts the reciprocal counteractions of XMAP215/TOGp and MCAK described in embryonic *Xenopus* systems (Tournebize *et al*, 2000).

Live analysis of TOGp-depleted cells indicates that type B multipolar spindles evolve gradually from bipolarity to multipolarity during prometaphase (Gergely *et al*, 2003). Such a gradual evolution is consistent with the idea that the type B multipolar spindles of CaMKII $\gamma$ - or TOGp-depleted cells do not involve nucleation from multiple centrosomes, as evidenced by co-staining of pericentrin and tubulin (Figure 1D; Holmfeldt *et al*, 2004). Based on the evolution and MCAK dependence of type B multipolar spindles, we have previously proposed that TOGp, from its centrosomal localization, has an essential role in protecting spindle MTs from cytosolic/centrosomal MCAK activity at the centrosome (Holmfeldt *et al*, 2004). An important prediction from this model is that excessive centrosomal and/or cytosolic MCAK activity should generate type B multipolar spindles, which was indeed shown to be the case in the present study (Figures 3 and 5). Based on the demonstrated interplay of CaMKII $\gamma$ , TOGp, and MCAK during mitosis, we have extended our previous model to the model depicted in Figure 7. In this



model, the type B multipolar spindle is viewed as a phenotype diagnostic for deficient control of cytosolic/centrosomal MCAK activity.

The present study suggests two independent levels of MCAK regulation involving CaMKII $\gamma$  and TOGp, in which CaMKII $\gamma$  and TOGp appear only strictly required for spindle bipolarity in so much as they are both essential to control cytosolic/centrosomal MCAK activity. Thus, co-depletion of MCAK restores formation of bipolar spindles and to an appreciative extent also cell proliferation in CaMKII $\gamma$ - or TOGp-depleted cells (Figure 2; Holmfeldt *et al*, 2004). Given that overexpression of either CaMKII $\gamma$ (c) or TOGp reciprocally suppresses generation of type B multipolar spindles in cells depleted of one of these two proteins (Figure 6), our data reveal a significant functional overlap between TOGp and CaMKII $\gamma$ , which can be linked to negative regulation of cytosolic/centrosomal MCAK activity. We also propose that the essential function of TOGp and CaMKII $\gamma$  for bipolar spindle formation is exerted from a centrosomal location, which in the case of TOGp is consistent with a prominent centrosomal localization in animal cells (Charrasse *et al*, 1998; Gergely *et al*, 2003). Interestingly, and consistent with our model, CaMKII has also been described as a spindle component that is enriched at the centrosome (Ohta *et al*, 1990).

Analysis of K562 leukemia cells reveals that the major part of overexpressed xMCAK protein resides in the cytosol and at spindle poles (Supplementary Figure S5). Our results demonstrate suppression of ectopic xMCAK activity by ectopic CaMKII $\gamma$ (c) on three distinct levels, namely (1) generation of type B multipolar spindles, (2) destabilization of the interphase MT array, and (3) generation of the mitotic type I bipolar spindle phenotype (Figures 4 and 5). As CaMKII $\gamma$ (c) does not suppress MT destabilization by Op18/stathmin or nocodazole (Supplementary Figures S2 and S3), phenotypic suppression cannot be attributed to nonspecific stabilization of MTs. While we propose that the first level reflects an action at the centrosome, it seems reasonable to assume that the second and third levels reflect suppression of MT plus-end-directed MCAK activity. We suggest that, similar to TOGp, CaMKII $\gamma$  has the potential to suppress both the MT plus-end- and minus-end-directed destabilizing activity of the cytosolic/centrosomal pool of MCAK. This interpretation is consistent with the observation that CaMKII $\gamma$  depletion is also associated with an increased frequency of prometaphase cells (Supplementary Figure S1), similar to what occurs upon overexpression of MCAK (Kline-Smith and Walczak, 2002).

The antagonistic activities of MCAK and XMAP215/TOGp at MT plus ends have been well established in the *Xenopus* system (Tournebize *et al*, 2000; Kinoshita *et al*, 2001). However, these studies do not provide any clear-cut mechanistic clue as to how centrosomal TOGp may protect the minus ends of centrosomal MTs from MCAK activity. On the other hand, in the case of CaMKII $\gamma$ -mediated MCAK suppression, it seems relevant that MCAK has been shown to be phosphorylated and inactivated by the Aurora B kinase (Andrews *et al*, 2004; Lan *et al*, 2004; Ohi *et al*, 2004). Thus, one simple model is that CaMKII $\gamma$  may activate Aurora B, or a related kinase, or by itself phosphorylate MCAK, and thereby inactivate MCAK. However, our present experimental data seem to exclude these possibilities since (1) coexpressed CaMKII $\gamma$ (c)

still suppresses an MCAK derivative lacking the important negative regulatory Aurora B phosphorylation sites (Figure 5) and (2) MCAK phosphorylated *in vitro* by CaMKII still retains full activity (Supplementary Figure S4). Moreover, we have also analyzed MCAK localization in cells that either overexpress or are depleted of CaMKII $\gamma$ (c), or that have been treated with the CaMKII inhibitor KN-93. These analyses did not indicate that CaMKII $\gamma$ -mediated control of MCAK activity involves alterations in the localization of centrosomal MCAK (Supplementary Figure S5; data not shown). Finally, our finding that CaMKII $\gamma$ (c) suppresses the mitotic phenotype of TOGp-depleted cells excludes the idea that CaMKII $\gamma$  operates through stimulation of the MCAK counteractive activity of TOGp. Thus, the mechanism behind CaMKII $\gamma$ -dependent suppression of cytosolic/centrosomal MCAK activity remains to be elucidated, but likely involves an intermediate in the pathway.

Depletion of MCAK alone or in combination with CaMKII $\gamma$  or TOGp in cultured human cells has no detectable effect on the density or appearance of the interphase MT array (Holmfeldt *et al*, 2004; data not shown), which suggest that the essential MT regulatory role of these proteins is restricted to the mitotic phases of the cell cycle. While centromeric MCAK is necessary for correction of chromosome attachment errors on spindles (Kline-Smith *et al*, 2004), there is presently no evidence that the cytosolic/centrosomal pool of MCAK exerts an essential function in mammalian cells. However, Kif2a, which is another kinesin-13 family member, has recently been shown to be necessary for bipolar spindle formation and depletion of Kif2a results in cells with monopolar spindles (Ganem and Compton, 2004). Based on subcellular localization and Si-RNA knockdown in human cells, the authors derived a model in which the activities of Kif2a and MCAK are spatially restricted such that Kif2a regulates MT minus ends at centrosomes while MCAK regulates MT plus ends at kinetochores. This model is indeed reminiscent of how the two kinesin-13 family members, Klp59C and Klp10A, function in chromosome segregation in *Drosophila* embryos (Goshima and Vale, 2003; Rogers *et al*, 2004). One apparent problem with the model is that a large fraction of all MCAK protein resides in both the cytosol and at the centrosome in cultured cells. Our current demonstration of TOGp and CaMKII $\gamma$ -dependent negative control of cytosolic/centrosomal MCAK provides a mechanism to keep MCAK inactive at those places, but leaves open the question of whether MCAK in those locations is activated at certain times during mitosis to act in spindle assembly.

## Materials and methods

### DNA constructs, transfection, and cell culture

The pMEP4 shuttle vector derivative directing inducible expression of the *Xenopus* ortholog of MCAK (xMCAK), originally termed XKCM1 (Walczak *et al*, 1996), human TOGp, human Op18/Stathmin, and the human constitutively active mutant of CaMKII $\gamma$ (c) (CaMKII $\gamma$ (c)) have been described previously (Melander Gradin *et al*, 1997; Holmfeldt *et al*, 2002). CaMKII $\gamma$ (c) was generated by substituting Thr-286 with Asp, which mimics autophosphorylation and results in a kinase that is substantially Ca<sup>2+</sup> independent (Hudmon and Schulman, 2002). To construct the N-terminally truncated xMCAK(187–732) derivative, a PCR approach was used with a 5' primer containing 21 nucleotides (nt) from the 5' untranslated region of Op18 (primer sequence: 5'-GAC CGG ATC CGG TAC CGT CGC TTG TCT TCT ATT CAC CAT GGA GCG ACT AGT

GGC AAC TCG G-3') and the T3 primer of pBluescript. The purpose of fusing the initiator Met-codon of xMCAK(187-732) with the untranslated 5' Op18 sequence was to increase protein expression in human cells. The PCR fragment was digested with *KpnI* and *XhoI* and used to replace the corresponding fragment of the pMEP4 vector.

Replicating EBV-based shuttle vectors for constitutive expression of shRNA that targets TOGp- and MCAK-specific RNA has been described (Holmfeldt *et al*, 2004). The targeting sequences (AA(19 nt)TT) used in the present study were as follows: CaMKII $\gamma$  (accession: L07044): (shRNA-CaMKII $\gamma$ ) CCT GCT GCT GGC GAG TAA A; scrambled version of shRNA-CaMKII $\gamma$  (shRNA-Co): GGA GGC TGA ACA TTC CGT C; MAP4 (accession: U19727): (shRNA-MAP4) GATAGTCCCAGCCAAGGAT. A BLAST search of the NCBI database ensured specific targeting of the cognate mRNA.

Single and double co-transfection using pMEP4 and shRNA derivatives, and subsequent selection of hygromycin-resistant cell lines over 4-6 days, were performed as described (Holmfeldt *et al*, 2004). Conditional expression/coexpression was induced from the hMTIIa promoter, which can be suppressed by cultivation in a specifically formulated medium and subsequently induced by 0.5  $\mu$ M Cd<sup>2+</sup> (Melander Gradin *et al*, 1997). The data from transfection cell lines shown in this report have been reproduced in at least three independent transfection experiments.

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## Immunoblotting and immunofluorescence

Quantification by immunoblotting, analysis of cellular MT content by flow cytometry, immunofluorescence analysis of spindles, and immunolocalization of centrosomes by pericentrin staining were performed as described previously (Holmfeldt *et al*, 2002, 2004). The NuMA antibody (clone A73-B/D12) and Alexa Fluor 488-conjugated anti- $\alpha$ -tubulin, was used for triple staining of NuMA, MTs and DNA using methanol fixed cells. Epifluorescence images were acquired on an Olympus CellR imaging station (Olympus-Biosystems) equipped with an inverted microscope (IX81; Olympus), an  $\times$ 100, 1.4 NA Planapochromat objective, and a cooled CCD camera (Orca ER; Hamamatsu Photonics).

## Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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