the abundance of *per* transcripts.

Light pulses rapidly induce expression of the *Neurospora* clock gene *frequency* (*frq*) (*29*). Although *frq* protein was not assayed, these results suggest that the regulation of *frq* transcription is the initial clock component modulated by photic stimuli. However, in *Drosophila* the initial clock-specific photoresponsive event is likely to be the degradation of TIM (*28*) and the disruption of the PER-TIM complex. Indeed, circadian fluctuations in both the abundance of PER and behavior can be generated from a presumably noncycling *per* transcript (*30*). Together, these observations suggest that post-translational autoregulatory loops (in addition to the possible contribution of the *per* and *tim* transcriptional feedback loop) (*11*, *16*) might participate in generating the PER and TIM biochemical oscillations.

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18. Similar results were obtained for flies light pulsed (approximately 1000 lux) from 5 to 60 min (*22*). This is consistent with the observation that similar phase-response curves are obtained for wild-type CS flies exposed to light pulses from 10 to 60 min in duration (*19*, *20*). Unless otherwise indicated, our results were obtained with 1-hour light pulses because these pulses produced the most consistent results (*22*). Light pulses were administered during the last dark cycle of LD to minimize desynchronization of the fly population during free-running conditions, as the biochemical analysis of PER requires material from many flies.
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21. Preparation of total fly head extract and visualization of PER by immunoblotting were done essentially as described (*7*, *8*).
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23. For each experiment, two sets of CS flies were entrained under identical LD conditions, and on the last dark period (day 4) one set received a light pulse at ZT15, followed by continued darkness. Heads of anesthetized flies were quickly isolated and immediately incubated in 4% paraformaldehyde at 4°C in the dark. All subsequent sectioning and staining steps were as described (*8*). To detect nuclei (Fig. 2B), we incubated slides with propidium iodide as described (*10*). Frozen head sections were observed under 1000× magnification with a Leica microscope interfaced with an image analysis system (Bioquant), and images were transferred to Adobe Photoshop software for printout.
24. For each time point, total RNA was extracted from ~50 μl of fly heads and ribonuclease protection assays were performed (*11*) with modifications described by Zeng *et al.* (*8*). The protected bands were quantified with a PhosphorImager from Molecular Dynamics.
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26. Head extracts were prepared in HP solution [defined in (*7*)] and either PER-HA or PER immunoprecipitated from equal amounts of homogenate as described (*7*). Protein immunoblotting in the presence of antibody to PER or antibody to TIM was as described (*7*), except that the antibody to TIM was diluted 1:500 in blocking solution that contained 0.02% Tween-20. The antibody to TIM used in this study was raised in rats with the use of a bacterially expressed fragment of TIM (amino acids 1 to 580) fused to polyhistidine as an immunogen (*28*).
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Abnormal Centrosome Amplification in the Absence of p53

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The centrosome plays a vital role in mitotic fidelity, ensuring establishment of bipolar spindles and balanced chromosome segregation. Centrosome duplication occurs only once during the cell cycle and is therefore highly regulated. Here, it is shown that in mouse embryonic fibroblasts (MEFs) lacking the p53 tumor suppressor protein, multiple copies of functionally competent centrosomes are generated during a single cell cycle. In contrast, MEFs prepared from normal mice or mice deficient in the retinoblastoma tumor suppressor gene product do not display these abnormalities. The abnormally amplified centrosomes profoundly affect mitotic fidelity, resulting in unequal segregation of chromosomes. These observations implicate p53 in the regulation of centrosome duplication and suggest one possible mechanism by which the loss of p53 may cause genetic instability.

The centrosome is a major microtubule-organizing center in eukaryotic cells and features prominently in mitosis, where it is required for the establishment of spindle bipolarity, spindle microtubule assembly, the establishment of the cleavage furrow plane, and balanced segregation of chromosomes (*1*). In addition, during interphase it nucleates and organizes the cytoplasmic microtubules, which leads to the redistribution of cellular organelles and the establishment of cellular polarity (*1*). The centrosome duplicates only once during each cell cycle; duplication begins near the G_1-S boundary, when replication of the centriole (the core component of centrosome) commences, and is completed in G_2 (*2*).

The *p53* tumor suppressor gene is frequently mutated in human and rodent tumors (*3*, *4*), and its loss or inactivation is correlated with genetic instability (*5*). The p53 protein has been shown to associate with the centrosome during interphase, but not during mitosis (*6*). To investigate

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whether the loss of p53 affects centrosome behavior, we studied primary MEFs derived from p53-deficient ($p53^{-/-}$) mice (*7*). We identified centrosomes by immunostaining with an antibody to γ-tubulin (anti–γ-tubulin), a well-characterized component of centrosomes (*8*) in all phases of the cell cycle. The microtubule-nucleating activity of centrosomes was examined by immunostaining with an antibody to β-tubulin.

We compared the centrosomes of $p53^{+/+}$ and $p53^{-/-}$ MEFs at the second cell passage. Control $p53^{+/+}$ MEFs at interphase contained one or two centrosomes juxtaposed to the nucleus (Fig. 1, A and A′, and Table 1), and at mitosis >97% of the cells displayed a typical bipolar array of antiparallel microtubules organized by two centrosomes at the poles (Fig. 1, B to E, B′ to E′, and Table 1). In contrast, >30% of the $p53^{-/-}$ MEFs at interphase contained more than two centrosomes (3 to 10 per cell) (Fig. 2, A to A″, and Table 1), and at mitosis >50% of the $p53^{-/-}$ MEFs contained spindles organized by multiple spindle poles (Fig. 2 and Table 1). The abnor-

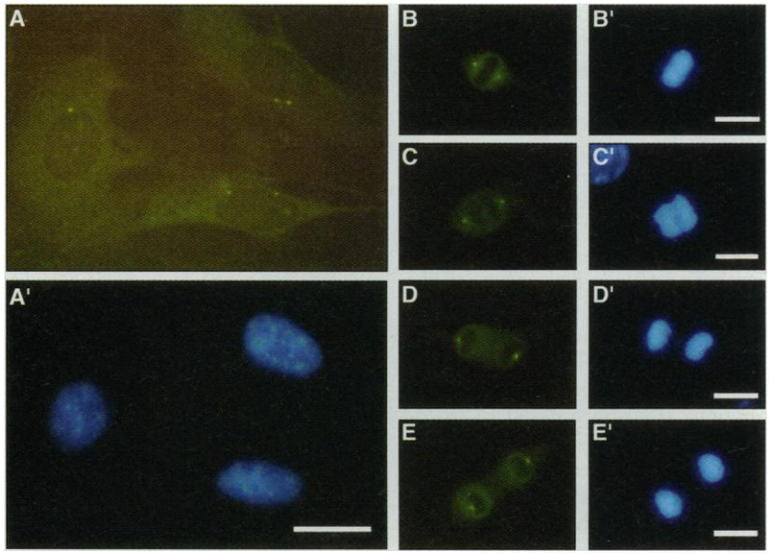

Fig. 1. Staining of γ-tubulin in $p53^{+/+}$ MEFs during interphase and mitosis. $p53^{+/+}$ MEFs were immunostained with anti–γ-tubulin. Antibody-antigen complexes were detected with fluorescein isothiocyanate (FITC)–conjugated antibody to rabbit immunoglobulin G (IgG) (green). Cells were also stained with 4′,6′-diamidino-2-phenylindole (DAPI) for visualization of DNA (blue) (*25*). (**A** and **A′**) Interphase; (**B** and **B′**) metaphase; (**C** and **C′**) anaphase; (**D** and **D′**) telophase; (**E** and **E′**) cytokinesis. (A) through (E), γ-tubulin staining; (A′) through (E′), DAPI staining. Scale bar, 10 μm.

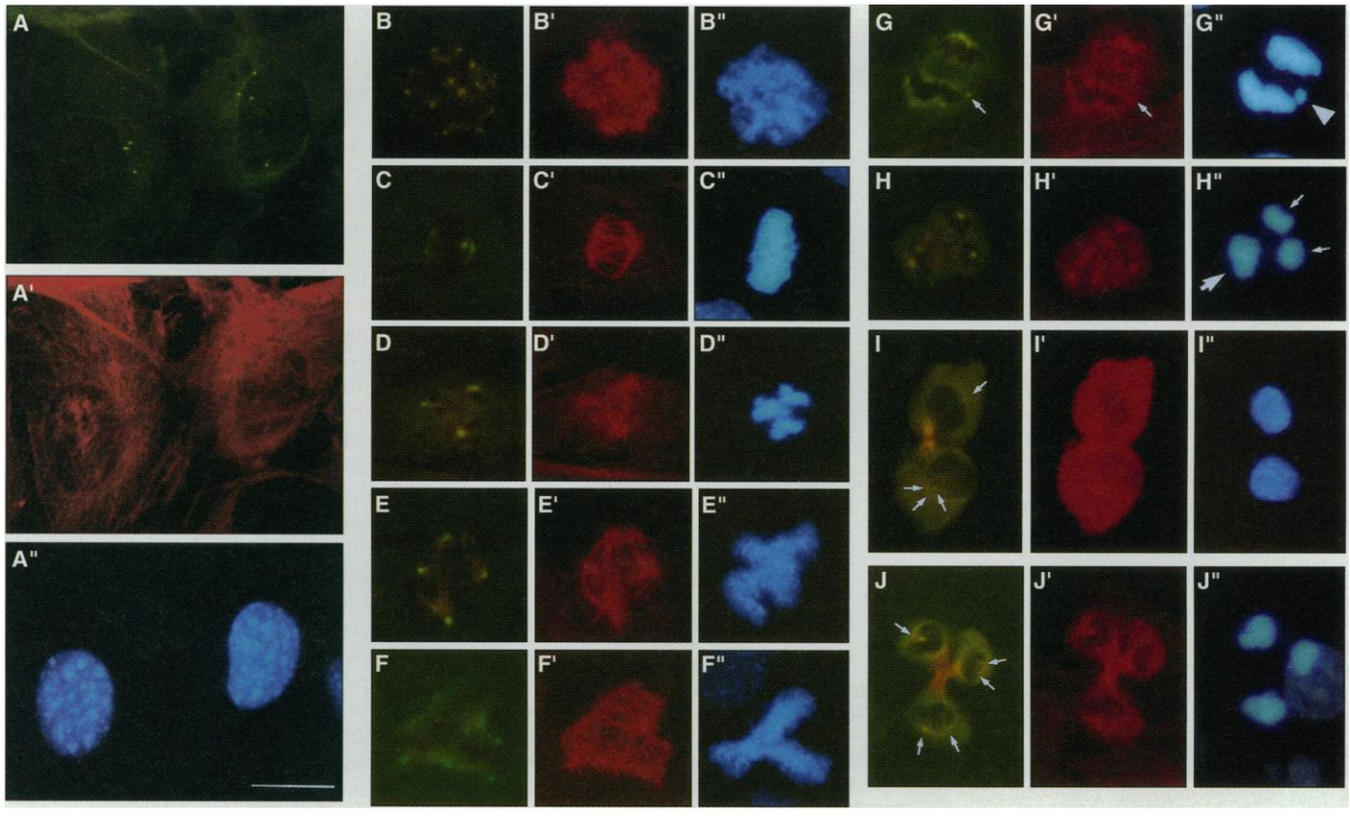

Fig. 2. Abnormal amplification of centrosomes in the absence of p53. $p53^{-/-}$ MEFs were immunostained with anti–γ-tubulin and anti–β-tubulin. Antibody-antigen complexes were detected with FITC-conjugated anti–rabbit IgG (for γ-tubulin, green) and rhodamine-conjugated anti–mouse IgG (for β-tubulin, red). Cells were also stained with DAPI for DNA dye (blue) (*25*). (**A** to **A″**) Interphase; (**B** to **B″**) prophase; (**C** to **C″**, **D** to **D″**, **E** to **E″**, **F** to **F″**) metaphase; (**G** to **G″**, **H** to **H″**) anaphase; (**I** to **I″**, **J** to **J″**) telophase (cytokinesis). (A) to (I), γ-tubulin staining; (A′) to (I′), β-tubulin staining; (A″) to (I″), DAPI staining. Arrows in (G) and (G′) indicate the centrosome localizing outside of the spindles formed in a bipolar fashion, and an arrow in (G″) indicates the corresponding chromosomes that failed to partition. Panel (H″) shows an unbalanced segregation of chromosomes; about twofold more chromosomes segregated to one pole (indicated by a large arrow) than those to the other two poles (indicated by small arrows). In panels (I) and (J), centrosomes that have been inherited in the daughter cells are indicated by arrows. Scale bar, 10 μm.

mally amplified centrosomes retained microtubule-nucleating activity (Fig. 2, B′ to J′) and localized to the bipolar axis in >90% of the cells in metaphase (Fig. 2C). This characteristic is most likely responsible for balanced chromosome segregation in most of the cells with multiple copies of centrosomes and generation of viable daughter cells (Fig. 2, I to I″). Much less frequently, three daughter cells were generated (Fig. 2, J to J″) when tripolar spindles were formed (Fig. 2, D to D″). To test whether the centrosome abnormalities are a common property of cells that have lost their tumor suppressor genes, we examined MEFs obtained from mice deficient in the retinoblastoma susceptibility gene (*Rb*) (9). No differences in the centrosome number in $RB^{-/-}$ and $RB^{+/+}$ MEFs were observed (Table 1), indicating that the abnormal centrosome amplification is specific to the $p53^{-/-}$ MEFs. We cannot exclude the possibility that other tumor suppressor gene or oncogene products may influence centrosome behavior.

In some cells, the presence of multiple centrosomes in the mitotic spindles had profound effects on chromosome segregation; the chromosomes did not partition during anaphase because they were captured by astral microtubules of one centrosome (or a few centrosomes) localized outside of the poles (Fig. 2, G to G″, indicated by arrows). In other cells, there was an unequal distribution of chromosomes to daughter cells (Fig. 2H″; see the legend to Fig. 1), perhaps caused by unequal numbers of centrosomes and the resultant differences in the mitotic force exerted at each spindle pole. When large numbers of centrosomes failed to localize at the poles in a bipolar fashion, the proper spindle apparatus did not form (Fig. 2, E to E″ and F to F″). These cells did not proceed further in mitosis and were eliminated during cell passage (*10*).

Quantitative analyses revealed that the abnormal amplification of centrosomes detected during interphase in $p53^{+/+}$ MEFs was ~2% versus ~33% in $p53^{-/-}$ cells and ~50% in $p53^{-/-}$ cells in mitosis (Table 1). However, there was no apparent increase in centrosome number in early- versus late-passage cells (Table 1), suggesting that there is a p53-independent selection against cells with more than three centrosomes. Thus, p53 loss may also deregulate the centrosome duplication itself.

To test this possibility, we cultured $p53^{+/+}$ and $p53^{-/-}$ MEFs (passage 2) for 60 hours with a minimal amount of serum (*11*) (serum-starved cells) and then serum-stimulated the cells for 15 hours. Analysis by flow cytometry and by 5′-bromo-2′-deoxyuridine (BrdU) incorporation showed that ~10% of the total cell population entered S phase during the period of serum stimulation in both $p53^{+/+}$ and $p53^{-/-}$ MEFs (*11*). Cells were stained with anti–γ-tubulin, and the number of centrosomes per cell was determined before and after serum was added (Fig. 3). Under serum-starved conditions, both $p53^{+/+}$ and $p53^{-/-}$ MEFs were cell cycle–arrested with DNA content that corresponded to G_0 or G_1 (*11*). However, whereas nearly 100% of $p53^{+/+}$ MEFs contained one centrosome (Fig. 3A), ~50% of the $p53^{-/-}$ MEFs had one centrosome, ~35% had two, and the reminder had more than two (Fig. 3C). The serum-starved cells with multiple centrosomes most likely inherited the centrosomes from a previous cell division (Fig. 2, I and J) or stopped dividing because of spindle abnormalities (Fig. 2, E and F). After serum stimulation, the number of $p53^{+/+}$ MEFs with one centrosome declined by only ~10% (Fig. 3B), consistent with cell cycle analysis (*11*) and previous studies showing that centrosome duplication begins near the G_1-S transition (*2*). In contrast, the number of $p53^{-/-}$ MEFs with one centrosome declined by ~40% (Fig. 3, C and D). Thus, after serum stimulation, centrosome amplification was observed in ~80% of the serum-starved $p53^{-/-}$ MEF population with one centrosome, although only 10% of the cells entered S phase (*11*). These results suggest that centrosome duplication may be initiated much earlier in the cell cycle in the absence of p53.

We also observed a substantial increase in the number of $p53^{-/-}$ MEFs with five or more centrosomes after serum stimulation (from 6 to 24.8% of the total cell population) (Fig. 3D). Given that the centrosome duplicates only once per cell cycle, the cells initially must have had three or more centrosomes in order to have five or more centrosomes after one cycle of centrosome replication. However, only 16.8% of the cell population contained three or more centrosomes under serum-starved conditions (Fig. 3C), suggesting that in the absence of p53, centrosome duplication may be initiated multiple times during a single cell cycle. By electron microscopy analysis, more than two centrioles were detected in individual cells, suggesting that the multiple centrosomes contained centrioles (*12*). We also found that all of the centrosomes were readily detected by an antibody to another centrosomal protein, pericentrin (*13*), and were capable of nucleating microtubules and of forming spindle poles (Fig. 2), suggesting that they arose through abnormal replication. However, it is possible that they are also generated by centrosome destabilization.

Table 1. Abnormal amplification of centrosomes in the absence of p53. The number of centrosomes was determined by immunostaining with anti-γ-tubulin (*23*). For each cell lineage, we examined more than 500 interphase cells and more than 200 mitotic cells. ND, not determined.

Cell lineage†	Percentage of cell population with the indicated number (*n*) of centrosomes			
	Interphase		Mitosis	
	n = 1 or 2	*n* ≥ 3	*n* = 2	*n* ≥ 3
	$p53^{+/+}$			
A	97.8	2.2‡	94.6	5.4
B	98.3	1.7‡	95.7	4.3
	$p53^{-/-}$			
T64	68.7	31.3	40.7	59.3
T65	68.2	31.8	45.5	54.5
T65*	63.8	36.2	ND	ND
	$RB^{+/+}$			
MEF 186-4	96.1	3.9‡	90.4	9.5
	$RB^{-/-}$			
MEF 186-3	95.8	4.2‡	90.8	9.2

†For all cell lineages except for T65* $p53^{-/-}$ MEFs (passage 20), passage 2 cells were examined. ‡In most $p53^{+/+}$ cells as well as $RB^{+/+}$ and $RB^{-/-}$ cells with abnormal centrosome amplification, *n* = 3 or 4. In the $p53^{-/-}$ cells, *n* ranged from 3 to ~10.

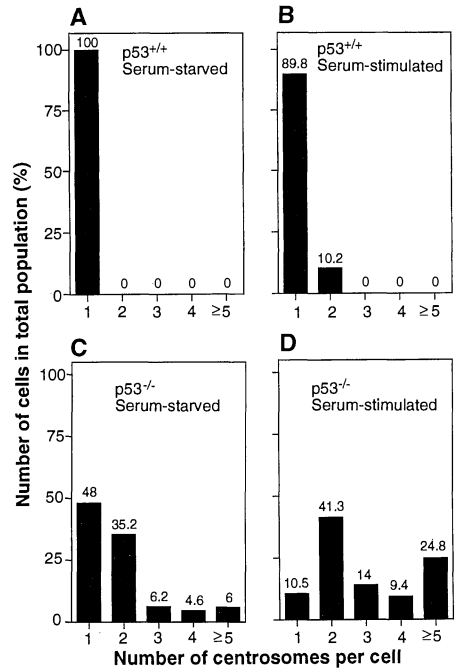

Fig. 3. Deregulation of the centrosome duplication cycle in the absence of p53. $p53^{+/+}$ and $p53^{-/-}$ MEFs were serum-starved for 60 hours (**A** and **C**), and then serum-stimulated for 15 hours (**B** and **D**). Cells treated in the same manner in parallel were analyzed by both flow cytometry and BrdU incorporation during the period of serum stimulation. These analyses showed that in both $p53^{+/+}$ and $p53^{-/-}$ MEFs, approximately 10% of cell population entered S phase (*11*). Cells before and after serum stimulation were immunostained with anti–γ-tubulin and then stained with DAPI. The number of centrosomes per cell in more than 1000 cells was determined by fluorescence microscopy.

The p53 protein has been implicated in cell cycle checkpoints (cell cycle arrest and cell death) at G_1-S (*14*) in response to DNA damage (*15*) or inappropriate oncogene expression (*16*), and at G_2-M (*17*), especially in response to microtubule poisons (*18*). Our observations raise the possibility that p53 may exert its checkpoint functions through the regulation of centrosome duplication. Multiple centrioles have been observed previously in pancreatic acinar cells by SV40 T antigen in mice (*19*). Our studies suggest that the multiple centrioles may arise through loss of p53 function mediated by the SV40 T antigen. In somatic cells, centrosome duplication requires the nucleus (or nuclear events) (*20*) and is controlled by the transcriptional activation of centrosome-specific genes at specific times in the cell cycle (*21*). Thus, conceivably, p53 has the potential to mediate its checkpoint functions as a transcription factor; p53 activates transcription of certain genes through binding to p53 response DNA elements (*22*) or represses transcription of many genes lacking p53 response elements (*23*). However, p53 has been shown to be physically associated with centrosomes (*6*), raising the possibility that it may directly influence centrosome activity. We propose that p53 actively participates in maintaining the stability of the genome through regulation of centrosome duplication or as a monitor that limits centrosome overproduction.

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11. Cells were grown in media containing 0.5% fetal bovine serum (FBS) for 60 hours (serum-starved), and then serum-stimulated by incubation in media containing 20% FBS for 15 hours. Flow cytometry and the BrdU incorporation assay were performed as described [K. Fukasawa *et al.*, *Oncogene* **10**, 1 (1995)]. Briefly, BrdU and 5′-fluoro-2′-deoxyuridine (10:1 ratio) were added to the serum-stimulation media. Flow cytometry of the serum-starved $p53^{+/+}$ and $p53^{-/-}$ MEFs showed that they were cell cycle–arrested with DNA content corresponding to the G_0 or G_1 phase of the cell cycle, which was identified by comparison with flow cytometry of the proliferating cells. BrdU incorporation was detected by a monoclonal antibody to BrdU, followed by incubation with peroxidase-conjugated antibody to mouse immunoglobulin G2a (IgG2a). The antibody-antigen complexes were visualized by reaction with a diaminobenzidine-hydrogen peroxide solution. Both methods showed that ~10% of $p53^{+/+}$ and $p53^{-/-}$ MEFs entered S phase (*24*). The γ-tubulin immunostaining and the 4′,6-diamidino-2-phenylindole (DAPI) staining were performed as described in (*25*).
12. K. Fukasawa and S. Rulong, unpublished data.
13. Centrosomes were also identified with antibody 5051, a human serum that recognizes pericentrin (*25*). In these experiments, the number of centrosomes per cell, as well as the percentage of $p53^{-/-}$ MEFs with three or more centrosomes, was similar to the results obtained with anti–γ-tubulin.
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26. Cells were grown on plastic microscopic slides at subconfluency in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 mg/ml) in an atmosphere containing 10% CO_2. The cells were then fixed with cold methanol for 10 min, rehydrated with phosphate-buffered saline (PBS), incubated with blocking solution (10% normal goat serum in PBS) for 1 hour, and probed with anti–γ-tubulin (dilution 1:150) for 1 hour. For $p53^{-/-}$ MEFs, incubation with anti–γ-tubulin antibody was followed by incubation with anti–β-tubulin monoclonal antibody [TUB2-1 (Sigma)] for 1 hour. The antibody-antigen complexes were detected with rhodamine-conjugated goat antibody to mouse IgG (Boerhinger Mannheim) for β-tubulin and fluorescein isothiocyanate (FITC)–conjugated goat antibody to rabit IgG (Boerhinger Mannheim) for γ-tubulin by incubation for 1 hour at room temperature. Cells were washed extensively with PBS after each incubation. Cells were then washed with tris-buffered saline and stained with DAPI.

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