Dynein Light Chain 1 (LC8) Association Enhances Microtubule Stability and Promotes Microtubule Bundling*

Received for publication, June 21, 2012, and in revised form, October 1, 2012 Published, JBC Papers in Press, October 4, 2012, DOI 10.1074/jbc.M112.394353

Jayant Asthana 1,2, Anuradha Kuchibhatla 1, Swadhin Chandra Jana 5, Krishanu Ray 7, and Dulal Panda 3

From the 1, 2 Department of Biosciences and Bioengineering, Indian Institute of Technology Bombay, Mumbai 400076, India and 5 Tata Institute of Fundamental Research, Homi Bhabha Road, Colaba, Mumbai 400005, India

Background: Dynein Light Chain 1 (LC8) has been shown to pull down tubulin subunits, suggesting that it interacts with microtubules. LC8 decorates microtubules in vitro and in Drosophila embryos, promotes microtubule assembly, and stabilizes microtubules both in vitro and in tissue-cultured cells. LC8 stabilizes microtubules.

Results: Data provide the first evidence of a novel MAP-like function of LC8.

Conclusion: LC8 stabilizes microtubules.

Significance: Data provide the first evidence of a novel MAP-like function of LC8.

Dynein light chain 1 (LC8), a highly conserved protein, is known to bind to a variety of different polypeptides. It functions as a dimer, which is inactivated through phosphorylation at the Ser-88 residue. A loss of LC8 function causes apoptosis in Drosophila embryos, and its overexpression induces malignant transformation of breast cancer cells. Here we show that LC8 binds to tubulin, promotes microtubule assembly, and induces the bundling of reconstituted microtubules in vitro. Furthermore, LC8 decorates microtubules both in Drosophila embryos and in HeLa cells, increases the microtubule stability, and promotes microtubule bundling in these cells. Microtubule stability influences a number of different cellular functions including mitosis and cell differentiation. The LC8 overexpression reduces the susceptibility of microtubules to cold and nocodazole-induced depolymerization in tissue-cultured cells and increases microtubule acetylation, suggesting that LC8 stabilizes microtubules. We also show that LC8 knockdown or transfection with inhibitory peptides destabilizes microtubules and inhibits bipolar spindle assembly in HeLa cells. In addition, LC8 knockdown leads to the mitotic block in HeLa cells. Furthermore, molecular docking analysis using the crystal structures of tubulin and LC8 dimer indicated that the latter may bind at α-β tubulin junction in a protofilament at sites distinct from the kinesin and dynein binding sites. Together, we provide the first evidence of a novel microtubule-associated-light chain-like function of LC8 that could explain its reported roles in cellular metastasis and differentiation.

Microtubule dynamics is involved in regulating the precise chromosome partitioning during anaphase (1), cell migration (2), cell shape (3), axonal growth cone guidance (4), and many other cellular phenomena (5, 6). Microtubule dynamics is precisely regulated in the cell through combined actions of several proteins, collectively known as microtubule-associated proteins (MAPs) (7). For example, MAP2, MAP4 (7), and Tau (8) associations with microtubule are known to stabilize microtubules, whereas that of Op18/stathmin and katanin (9, 7), to name a few, destabilize the microtubules. The loss of microtubule stability in the axons due to Tau hyperphosphorylation (9, 10) or the loss of certain MAP function causes severe neurodegeneration and axonal transport defects. The mutations in futsch/22C10, which codes for a MAP1B-like protein, affect dendritic and axonal development (11), whereas loss of stathmin destabilizes the neuromuscular junctions (12) in Drosophila. Several other MAPs such as EB1 and CLIP-170 track the dynamic plus-ends of microtubules and assist microtubule growth (13). Deletion of Mal3, an EB1 homologue, causes mitotic delay in fission yeast (14), and loss of CLIP-170 affects metaphase plate formation and leads to the activation of check point proteins (15). Often microtubule stabilization in the cells is indicated by specific MAP association (7, 16). Interestingly, we found that the suppression of microtubule dynamics in tissue-cultured cells is associated with an increased localization of the 8-kDa dynein Light Chain 1 (LC8), a component of cytoplasmic dynein, on microtubules (17).

LC8 contains 89 amino acid residues, and it is a highly conserved essential protein (18–26), with a sequence identity of 94% between Drosophila LC8 and human LC8 (27). LC8 is also known as DLC1 (28, 29). It was originally identified as the smallest subunit of cytoplasmic dynein (LC8) in Chlamydomonas (18–20), shown to act independently as the protein inhibitor of nNOS (PIN) in mammals (21), as an essential light chain of myosin V (DynL1) (22), and binds to many other proteins independent of dynein (23, 24). LC8 is ubiquitously expressed in all eukaryotic cells (25, 26). Deletion of the LC8 gene in Drosophila (dlc1/ctp) causes widespread apoptosis and early embryonic lethality (30), and LC8 loss or inhibition in tissue...
cultural cells also induces apoptosis (31). LC8 is known to function as a dimer (32), essentially linking two key subunits within a multiprotein complex (33, 34). The p21-activated kinase (Pak-1)-dependent phosphorylation of Ser-88 residue converts it into a monomer, which adversely affects the cell survival (25, 26). Apart from its traditionally attributed roles in the functioning of dynein and myosin V (20, 22), this small protein is expected to have several independent roles in the tissue (35). In this context, two specific evidences are worth considering. First, the overexpression of LC8 in normal breast cancer and fibroblast cell lines increases survival and cell growth (25), and second, it affects macropinocytosis in these cells (26). Although both these functions are attributed to the LC8 role in dynein and myosin V-related activities in the cell, the possibility of an independent LC8 function could not be ruled out.

Among many other proteins, LC8 was also found to pull down tubulin subunits from the rat brain extracts (36). In addition, Drosophila LC8 immunostaining was found to highlight microtubule filaments in the terminally differentiated cells in Drosophila (30). These led us to investigate a possible LC8 role in regulating microtubule dynamics. For the first time we found that a direct interaction between LC8 and tubulin/microtubules increased the assembly of purified tubulin, increased microtubule stability, and induced their bundling in vivo. This established a new MAP-like function of the protein in vivo.

**EXPERIMENTAL PROCEDURES**

**Materials**—Mouse monoclonal anti-α-tubulin IgG, alkaline phosphatase-conjugated anti-mouse IgG, FITC (fluorescein isothiocyanate)-conjugated anti-mouse IgG, bovine serum albumin, FITC, and Hoechst 33258 dye were purchased from Sigma. Anti-mouse IgG-Alexa 568 conjugate, Lipofectamine 2000, Lipofectamine RNAiMax, and Opti-MEM I were purchased from Invitrogen. Mouse monoclonal anti-LC8 IgG was obtained from Abcam (Cambridge, MA) and Biokline (Chennai, India). Fetal bovine serum was purchased from Biowest (Nuvaille, France). All other reagents were of analytical grade and were obtained from either Sigma or Himedia (Mumbai, India). Dim (DVSTQTPSP) and nNOS (MKGIQVDRDLDGKSHKA) peptides were synthesized.

**Isolation of Tubulin**—Goat brain tubulin was isolated by two cycles of polymerization and depolymerization in the presence of 1 M glutamate and 10% DMSO (37, 38).

**Purification of Recombinant LC8—His<sub>6</sub>-tagged Drosophila LC8 ORF cloned into the NdeI and BamHI sites of pET-14b was expressed in Escherichia coli B/L21 (DE3) cells. The transformed cells were grown at 37 °C in LB media containing 100 μg/ml ampicillin to an A<sub>600</sub> of 0.8 from an overnight culture. Then, His<sub>6</sub>-LC8 expression was induced by the addition of 0.6 mM isopropyl-β-thio galactopyranoside, and the culture was incubated at 37 °C for an additional 6 h. The cells were harvested by centrifugation at 10,000 × g for 4 °C for 10 min. The pelleted cells were resuspended in lysis buffer (20 mM Tris-HCl (pH 8.0), 10 mM imidazole, 200 mM NaCl) containing leupeptin, pepstatin, PMSF, 1% Triton X-100, and lysozyme. The cells were further lysed by sonication. The lysate was centrifuged at 17,000 × g for 1 h at 4°C. The crude extract was purified by affinity chromatography on nickel-nitrioltriacetic acid resin by elution with 250 mM imidazole. The LC8-His tag protein was dialyzed against 20 mM Tris buffer (pH 8.0) containing 200 mM NaCl and 2 mM dithiothreitol to remove the imidazole. Furthermore, the His tag was removed by digesting His-tagged-LC8 protein with 2 units/ml thrombin at 37 °C for 3 h. The reaction was quenched by adding 2 mM PMSF. The thrombin and His tag impurities were removed by passing through a benzimidazole column containing 20 mM Tris buffer (pH 8.0) and then by dialyzing against 20 mM Tris buffer (pH 8.0). The histidine tag cut of His-tagged-LC8 was confirmed by a 15% SDS-PAGE and Axima-CFR MALDI-TOF-MS by comparing with His-tagged-LC8.

**FITC Labeling of LC8**—LC8 possesses several lysine residues, and one or more of the lysine residues can be covalently modified using FITC. LC8 and FITC were incubated in the ratio of 1:5 in 50 mM sodium phosphate buffer (pH 8.0) for 4 h on ice. Then the protein was centrifuged at 88,000 × g for 10 min to remove any aggregate formed. Free FITC was removed from that of the LC8-bound FITC by dialyzing the reaction mixture against 20 mM Tris buffer (pH 8.0) followed by a P6-gel filtration column, which was equilibrated with 20 mM Tris buffer (pH 8.0). The concentration of LC8 was estimated by the method of Bradford (39), and the concentration of LC8-bound FITC was determined from the absorbance at 495 nm using a molar extinction coefficient 77,000 M<sup>-1</sup> cm<sup>-1</sup>. The incorporation ratio of FITC per LC8 monomer was found to be 0.8.

**Size Exclusion Chromatography**—Tubulin (12 μM) and LC8 (20 μM) were mixed in 25 mM PIPES buffer (pH 6.8) and 300 mM NaCl and incubated on ice for 20 min. The complex was loaded onto a Sephadex G-100 column (50 cm) pre-equilibrated with 25 mM PIPES buffer (pH 6.8) and 300 mM NaCl. The complex was eluted using the same buffer at a flow rate of 0.5 ml/min, maintained by peristaltic pump (Amersham Biosciences, GE Healthcare). Sixty-five fractions, each having volume 0.5 ml, were collected. The protein in the eluted fractions was detected using the Bradford method (39). The elution profile of the samples (only tubulin, tubulin-LC8 complex, and only LC8) was obtained by plotting the optical density against the elution volume graph. The peak fractions of the samples were also analyzed by 18% SDS-PAGE and stained with Coomassie Brilliant Blue R. The stoichiometry of LC8-tubulin binding was determined by loading the peak fraction from the tubulin-LC8 complex on SDS-PAGE with known concentrations of the LC8 and tubulin in the same gel. After staining the gel, the band intensities were calculated by using ImageJ software.

**Determination of Dissociation Constant for LC8 and Microtubule Binding**—Tubulin (20 μM) was polymerized in the buffer containing 25 mM PIPES (pH 6.8), 3 mM MgCl<sub>2</sub>, 1 mM EGTA, 20 μM taxol, and 1 mM GTP at 37 °C for 30 min. The polymerized sample was diluted 5-fold in buffer A (25 mM PIPES, 3 mM MgCl<sub>2</sub>, 1 mM EGTA, and 2 μM taxol), and different concentrations of LC8 (0.5, 1, 2, 4, 5, 6, 8, and 10 μM) were added to the mixtures. The samples were incubated for 10 min at room temperature, then centrifuged at 88,000 × g for 30 min. The pellet...
fractions were separated from the soluble fractions. The pellets were dissolved and loaded on 18% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue R. The band intensities were calculated by ImageJ software. The band intensities were fitted into the equation, $I_x = I_{\text{max}} \times C/(K_d + C)$, where $I_x$ is the band intensity at any concentration of LC8, $I_{\text{max}}$ is the maximum band intensity, and $C$ is the concentration of LC8. The dissociation constant ($K_d$) for LC8 to microtubule was calculated using the Graph Pad Prism 5 software (Graph Pad Software).

**Light Scattering Assay**—Tubulin (15 μM) was polymerized in the absence and presence of different concentrations (4 and 6 μM) of LC8 in buffer containing 25 mM PIPES (pH 6.8), 3 mM MgCl₂, and 1 mM EGTA. After the addition of 1 mM GTP, the reaction mixture was immediately transferred to 37 °C, and the kinetics of tubulin assembly was monitored by 90° light scattering. The polymerization was continued for another 20 min. The polymer fraction was probed with the antibody against tubulin and LC8. Images were captured using a perfusion Tecnai-G² 12 electron microscope (Philips, OR).

**In Vitro Immunostaining**—Tubulin (10 μM) was polymerized in the absence and presence of 4 μM LC8 in buffer containing 25 mM PIPES (pH 6.8), 3 mM MgCl₂, 1 mM EGTA, and 1 mM GTP at 37 °C for 30 min. Tubulin (10 μM) was also polymerized in the presence of 2 μM taxol in the same buffer, and the samples were adsorbed on the Formvar carbon-coated copper grids. The grids were subsequently subjected to negative staining by 2% uranyl acetate solution. The samples were examined using a FEI Tecnai-G² 12 electron microscope (Philips, OR).

**Fluorescence Microscopy**—Tubulin (7 μM) was incubated in the absence and presence of different concentrations of FITC-LC8 in buffer containing 25 mM PIPES (pH 6.8), 6 mM MgCl₂, 1 mM EGTA for 20 min on ice. To this mixture 1 mM GTP was added, and tubulin was polymerized at 37 °C for 30 min. These samples were placed on a coverslip and observed using a fluorescence microscope (Nikon Eclipse TE2000-U, Tokyo, Japan). The images were captured using a Cool SNAP-Pro camera and processed using Image-Pro Plus Version 5.0 software.

**Dilution Induced Disassembly Assay**—Tubulin (20 μM) was polymerized in buffer containing 25 mM PIPES (pH 6.8), 10% DMSO, 3 mM MgCl₂, and 1 mM GTP for 30 min. Preformed microtubules were diluted 10 times with warm buffer containing 25 mM PIPES, 3 mM MgCl₂, and 1 mM GTP in the absence and presence of 2 and 4 μM LC8 and incubated for an additional 5 min at 37 °C. The samples were then centrifuged at 88,000 × g for 20 min at 30 °C. The pellet obtained was dissolved in PIPES buffer and quantified on 12% SDS-PAGE using ImageJ software.

**Cell Culture**—HeLa cells were grown and maintained at 37 °C in a humidified atmosphere with 5% CO₂ and 95% air as previously described (17).

**Transfection of HeLa Cells with LC8 and S88E-LC8 Plasmids**—HeLa cells were seeded at a density of 2 × 10⁵ cells/ml in antibiotic free media for 24 h. The cells were then transfected with pCDNA3.1LC8 and pCDNA3.1S88E-LC8 plasmids (25) (a kind gift from Dr. Rakesh Kumar, MD Anderson Cancer Centre, UT Houston) using Lipofectamine 2000 and Opti-MEM I-reduced serum medium following the manufacturer’s protocol. After transfection, the cells were selected in the presence of G418 (0.26 mg/ml).

**Cold Treatment of LC8-transfected HeLa Cells**—Cells stably expressing LC8 and S88E-LC8 were seeded on glass coverslips in 24-well plates at a density of 0.5 × 10⁵ cells/ml and grown for 24 h. After that the cells were kept on ice for 10, 20, and 60 min and then fixed for immunostaining using 3.7% formaldehyde for 30 min at 37 °C followed by methanol treatment for 20 min. Cells were stained with tubulin antibody (1:300). Alexa Fluor-labeled secondary antibody (1:400) was used.

**Microtubule and LC8 Coimmunostaining in Drosophila Embryo**—The embryos were fixed with 4% paraformaldehyde in the microtubule stabilization buffer (0.1 M PIPES, 2 mM EGTA, 1 mM MgSO₄ (pH 6.9)) containing 0.2 μM taxol and then washed with the same buffer containing 0.3% Triton X-100 for 15 min. This helped to stabilize and fix dynamic microtubules in the tissue and to extract all the cytoplasmic (soluble) LC8 and tubulin. Tubulin and LC8 were stained using respective antibodies.

**Colocalization of LC8 with Microtubules in HeLa Cells**—HeLa cells were seeded at a density of 0.5 × 10⁵ cells/ml. After 24 h of incubation, cells were grown in the presence and absence of either 20 μM taxol or 100 nM nocodazole for a further 24 h. Cells were fixed and permeabilized with methanol and 100 mM EGTA. Cells were then processed and stained for tubulin and LC8. Images were captured using a FV500 confocal laser-scanning microscope (Olympus, Tokyo, Japan).

**Nocodazole Treatment of LC8-transfected HeLa Cells**—Cells were seeded on glass coverslips in 24-well plates at a density of 0.5 × 10⁵ cells/ml and grown for 24 h. The cells were treated with two different concentrations of nocodazole (300 and 500 nM) and incubated for an additional 24 h. Then the cells were fixed, and tubulin staining was performed as described above.

**Analysis of Polymeric Mass of Tubulin in Transfected and Untransfected HeLa Cells**—Polymeric and soluble tubulin fractions of untransfected and LC8- and S88E-LC8-transfected HeLa cells were prepared, and Western blotting was performed as described earlier (17, 41).

**Analysis of LC8 in Polymeric Fraction of Tubulin**—Cells were incubated with vehicle (0.1% DMSO), nocodazole, or taxol for 24 h. Polymer and soluble fractions of tubulin were separated, and the polymer fraction was probed with the antibody against tubulin and LC8 (41).

**Treatment of HeLa Cells with LC8 siRNA and Nocodazole**—HeLa cells were transfected with LC8 siRNA (target sequences for LC8 are 5′-GGACUGCACCUAAUUCC-3′ and 5′-GGAAUUAGGCGAGUCC-3′, Eurogentec) using Lipofectamine RNAiMax and Opti-MEM I-reduced serum.
LC8 Stabilizes Microtubules

medium. Then the cells were treated with 100 nM nocodazole and grown for 24 h, and subsequently, cells were stained for tubulin. Only LC8 siRNA-treated and nocodazole-treated cells were taken as controls for the experiment. Similarly, the HeLa cells were treated with LC8 siRNA, and mitotic microtubules were stained using tubulin antibody. The phospho-histone H3-positive cells were counted as mitotic cells. The mitotic index was calculated by dividing the number of mitotic cells by the total number of cells.

Treatment of HeLa Cells with Bim and nNOS Peptides and Nocodazole—HeLa cells were transfected with Bim (DKSTQTPSP) and nNOS (MKGIQVDRDLGKSHKA) peptides using Lipofectamine 2000 and Opti-MEM I-reduced serum medium. Then the cells were treated with 100 nM nocodazole, and tubulin staining was performed.

Modeling the Tubulin-LC8 Complex—A 3.5 Å resolution crystal structure of α/β tubulin dimer proteins from Bos taurus (PDB code 1JFF), a 3.8 Å resolution crystal structure of α and β tubulin tetramer proteins from Ovis aries (PDB code 3HKC), and a high resolution solution structure of the LC8 monomer and dimer proteins from Drosophila melanogaster and Rattus norvegicus, respectively (PDB codes 1RHW and 1F96), were used for making the model for the LC8-tubulin complex. To make the model, the LC8 monomer and dimer was docked on to α/β tubulin dimer by using GRAMM 1.03, independently (42). We used the generic docking with grid step 6.5 Å, grid size 64 Å. The ligand was rotated at 10° intervals. The energy score for repulsion was 6.5, and that for attraction was 0. The 40 docked structures, among the top 1000 best score matches, were selected and analyzed using WHATIF (43) and CSU (44) servers. Once again, the models were subjected to energy minimization using Swiss-PdbViewer V4.0.3, and the best docked model was selected on the basis of the lowest calculated stabilization energy (∆E) and the highest numbers of probable salt bridges, hydrophobic interactions, and hydrogen bonds between the proteins. In the same manner, the tubulin trimer was docked to obtain an independent LC8 α/β/α tubulin model. To further test the specificity of the α/β-tubulin/dimer-LC8 model and the interaction between phosphorylated LC8 and α/β-tubulin, few modified forms of LC8, such as LC8 monomer and phosphomimetic mutant LC8 (S88E) monomer and dimer were docked to the α/β-tubulin.

RESULTS

LC8 Colocalizes with Microtubules in the Drosophila Embryo and in Cultured HeLa Cells—The study was initiated with the following preliminary observations. LC8 immunostaining highlighted microtubules in the early, syncitium-stage Drosophila embryos. LC8 was found to decorate microtubules in both the interphase (Fig. 1A, upper panel) and on mitotic spindles (Fig. 1B). A similar LC8 staining in the mature nurse cells was reported earlier in Drosophila ovary (30). ddc11 isoform carries a deletion covering the entire LC8 gene, and cells of the homozygous embryos survive until the onset of gastrulation due to the maternal contribution. Anti-LC8 staining and its localization on the microtubules were substantially reduced, and most of the microtubule filaments were destabilized in the ddc1 exc1 homozygous embryos (Fig. 1A, lower panel). LC8 was also found to colocalize with microtubules in HeLa cells (Fig. 1C). In addition, HeLa cells incubated with 20 nM taxol increased LC8 localization on microtubules as compared with the control, and 100 nM nocodazole treatment retained only LC8-associated microtubules in the cells (Fig. 1C). The concentrations of taxol and nocodazole used are known to suppress dynamic instability of microtubules in tissue culture cells (45). These results indicated that either LC8 binds more efficiently to microtubules with suppressed dynamic instability or the binding of LC8 stabilizes microtubules dynamics.

LC8 Binding Enhances Tubulin Assembly, Stabilizes Microtubules, and Promotes Their Bundling—To test this hypothesis, purified goat brain tubulin was assembled in the presence of 5 μM taxol, and then, the purified, recombinant LC8 was added to the preformed polymers. This resulted in the formation of thick bundles of microtubules (Fig. 2A). Immunostaining of these preparations showed that LC8 was localized all along the length of the microtubules.
of the microtubules, and the intensity of LC8 was more on thick microtubule bundles. The interaction between purified tubulin and LC8 was further analyzed by size-exclusion chromatography in the presence of 300 mM sodium chloride (Fig. 2B). LC8, when mixed with tubulin, eluted together at 11 ml in G-100 column chromatography, whereas LC8 alone eluted at 21.5 ml and purified tubulin at 11.5 ml (Fig. 2B) as evident from the SDS page analysis of the peak fractions (Fig. 2C). The stoichiometry of binding of LC8 to tubulin was determined to be 1.1 ± 0.03 LC8 per tubulin dimer (Fig. 2C). Western blot analysis of the tubulin polymeric fraction isolated from the tissue culture cells further indicated that more LC8 binds to microtubules in the cells treated with either 100 nM nocodazole or 20 nM taxol, as compared with the control cells (Fig. 2D). There was an ∼1.6 and 1.8-fold increase in the amount of LC8 bound with polymeric tubulin fraction in the taxol- and nocodazole-treated cells, respectively. Furthermore, we found that the addition of FITC-LC8 into the microtubule assembly milieu produced uniformly fluorescent microtubule bundles, suggesting that the FITC-LC8 decorated all along the length of microtubules (supplemental Fig. S1).

The assembly kinetics of tubulin was monitored by light scattering at 350 nm. LC8 increased the light scattering signal of tubulin assembly in a concentration-dependent manner, suggesting that it promotes the assembly of tubulin (Fig. 3A). However, LC8 did not promote the assembly of tubulin in the presence of GDP (Fig. 3A). The addition of 6 μM carbonic anhydrase to the polymerization mixture did not increase the light scattering intensity of the tubulin assembly (Fig. 3A), suggesting that the increase in the tubulin assembly was specific to LC8. Furthermore, electron microscopic analysis suggested that LC8 (4 μM) increased the assembly of tubulin and induced bundling of microtubules (Fig. 3B). Under the conditions used, tubulin (10 μM) produced only few thin microtubules (Fig. 3B), whereas several microtubules were observed per field of view when 2 μM taxol was included in the assembly mixture (Fig. 3C). LC8 (4 μM) was found to increase microtubule assembly and also to induce the bundling of microtubules in the presence of taxol (Fig. 3C), suggesting that the LC8 binding site on tubulin is different from that of the taxol. The dissociation constant for the binding of LC8 to microtubules was determined to be 2.6 ± 0.3 μM (supplemental Fig. S2, Fig. 3D). Furthermore, LC8 suppressed the dilution-induced disassembly of preformed microtubules (Fig. 3E). For example, 4 μM LC8 increased the amount of tubulin pelleted by 4-fold as compared with its absence, suggesting that LC8 stabilizes microtubules and protects them against dilution-induced disassembly.

**Overexpression of LC8 Stabilizes Microtubules in HeLa Cells**—LC8 is a dimeric protein, which dissociates into monomers either at low pH (46) or by phosphorylation at the Ser-88 residue (29). S88E-LC8 is a phosphomimetic form of LC8 and is reported to be nonfunctional (29). The role of LC8 in the assembly and stability of microtubules was examined by expressing wild type (WT) and S88E-LC8 constructs in HeLa cells. The level of overexpression of S88E-LC8 and LC8 proteins in HeLa cells is shown in Fig. 4A. The quantification of the data indicated that S88E-LC8 and LC8 were overexpressed by ∼2 and ∼3-fold, respectively, as compared with the control cells. The S88E-LC8-transfected cells had a similar microtubule network as that of the control untransfected cells, whereas the overexpression of WT variant resulted in an apparent increase in the level of microtubules (Fig. 4B). Some of the filaments appeared brighter and thicker (Fig. 4B), implying that the presence of LC8 may also promote microtubule bundling in the cell. Western blot analysis also indicated that there was an increase in the polymeric fraction of tubulin with a concomitant decrease in its soluble fraction in LC8-overexpressing cells as compared with both the untransfected and S88E-LC8-expressing cells (Fig. 4C). The overexpression of LC8 increased the amount of polymerized tubulin by 60% than the controls, viz. the untransfected and the S88E-LC8-expressing cells. Tubulin acetylation is a marker of stable microtubules in the cells (47). LC8 overexpression increased the level of acetylated tubulin in HeLa cells.
Western blotting for the levels of acetylated tubulin was done in control and transfected cells (supplemental Fig. S3). The levels of tubulin and acetylated polymer levels were normalized with respect to control. The amount of polymerized tubulin was found to be increased by 48 ± 2% in LC8-overexpressing cells as compared with the control cells, whereas it remained unchanged in S88E-LC8-overexpressing cells (supplemental Fig. S3B). As compared with the control cells, the level of acetylated tubulin was found to increase by 10 ± 8 and 90 ± 13% in S88E-LC8- and LC8-overexpressing cells, respectively.

**FIGURE 3.** LC8 enhanced the assembly of purified brain tubulin in vitro. A, LC8 promoted the assembly of tubulin. Tubulin (15 μM) was polymerized in 25 mM PIPES buffer (pH 6.8) containing 3 mM MgCl₂, 1 mM EGTA, and 1 mM GTP at 37 °C in the absence (■) and presence of 4 (▲) and 6 μM (×) LC8. Under similar condition tubulin was also polymerized in presence of 10 μM (▲) taxol and 6 μM (▲) carbonic anhydrase. Tubulin (15 μM) was also polymerized in the buffer containing 25 mM PIPES buffer (pH 6.8) containing 3 mM MgCl₂, 1 mM EGTA, and 1 mM GDP (●) in the presence of 6 μM LC8. Only 6 μM LC8 (●) was shown in the figure. B and C, electron micrographs show the effects of LC8 on tubulin polymerization. Tubulin (10 μM) was polymerized without or with 4 μM LC8 (B) and (in the presence of 2 μM taxol without or with 4 μM LC8 (C). Scale bar, 500 nm. D, the band intensities of LC8 co-sedimented with microtubules were plotted against LC8 concentration. a.u., absorbance units. E, LC8 prevented the dilution-induced disassembly of microtubules. Preformed microtubules were diluted 10 times in warm 25 mM PIPES buffer (pH 6.8) containing 3 mM MgCl₂ and 1 mM GTP without and with 2 and 4 μM LC8. The reaction mixtures were incubated for an additional 5 min at 37 °C. Polymers were sedimented at 88,000 × g for 20 min at 30 °C. The sedimeted polymers were dissolved in PIPES buffer and ran on a 12% SDS-PAGE.
The data suggested that LC8 overexpression stabilizes microtubules in tissue-cultured cells and may promote microtubule bundling.

The effect of LC8 overexpression on microtubule stability was further examined either by challenging the cells with 300 and 500 nM nocodazole or by cold treatment. The 300 nM nocodazole treatment substantially reduced microtubules in control and S88E-LC8-overexpressing cells, whereas microtubules were less affected in LC8-overexpressing cells (Fig. 5A). The effects were even more severe in the presence of 500 nM nocodazole (Fig. 5A). Western blot analysis also suggested that microtubules of the LC8-overexpressing cells were more resistant toward nocodazole treatment (Fig. 5B). In the presence of 300 nM nocodazole, LC8-overexpressing cells had ~30% more polymer tubulin than that of the control and S88E-LC8-overexpressing cells, respectively. The polymer to soluble
LC8 Stabilizes Microtubules

tubulin ratio was determined to be 0.93 ± 0.1, 0.88 ± 0.1, and 1.26 ± 0.2 in control and S88E-LC8- and LC8-overexpressing HeLa cells, respectively (supplemental Fig. S4A). A similar result was observed after the cold treatment. Most of the microtubules in the untransfected and S88E-LC8-expressing cells were found to be depolymerized upon 10 min of incubation on ice. However, the depolymerization was not so apparent in the LC8-overexpressing cells (Fig. 5C).

The Depletion as Well as the Pharmacological Inhibition of LC8 Destabilized Interphase Microtubules in HeLa Cells—The role of LC8 in the organization and stability of both interphase and mitotic microtubules in HeLa cells was analyzed by depleting the protein using siRNA. There was a marked decrease in the level of LC8 protein after siRNA treatment, whereas the level of actin did not change (Fig. 6A). The organization of interphase microtubule networks were visibly similar in both LC8-depleted and control HeLa cells (Fig. 6B), indicating that the depletion did not induce a noticeable effect on the interphase microtubule network. Moderate depolymerization of interphase microtubules was noticed in HeLa cells treated with a low concentration (100 nM) of nocodazole (Fig. 6B). When LC8 siRNA was used in combination with 100 nM nocodazole, it produced a significant depolymerization of the interphase microtubules, indicating that the depletion of LC8 reduces the stability of the microtubule network. A significant decrease in the amount of polymerized tubulin was also evident from the Western blot analysis in cells treated with a combination of LC8 siRNA and 100 nM nocodazole (Fig. 6C). The ratio of polymer to soluble tubulin was found to be 1.2 ± 0.1 and 0.88 ± 0.04 in control and 100 nM nocodazole-treated cells, whereas in cells treated with a combination of LC8 siRNA and 100 nM nocodazole, it was found to be 0.5 ± 0.1 (supplemental Fig. S4B). LC8 siRNA-treated cells had a similar amount of polymeric/soluble tubulin (1.2 ± 0.1) as control. Cells treated with the combination of LC8 siRNA and 100 nM nocodazole showed 47% less polymeric tubulin as compared with the control, whereas only 100 nM nocodazole-treated cells had 12% less polymeric tubulin than the control (Fig. 6C). The combined use of LC8 siRNA and 100 nM nocodazole had 40% less polymeric tubulin than that of the cells treated with only 100 nM nocodazole. LC8 siRNA-treated cells had similar amount of polymeric tubulin as control (Fig. 6C).

LC8 binds to Bim and nNOS through specific peptide motifs (25, 31, 32) that are known to engage LC8 by different mechanisms (32). Therefore, LC8 binding peptides obtained from Bim and nNOS were used to inhibit LC8 function, and its effects on microtubule network were analyzed. Treatments with 50 μM Bim or 50 μM nNOS peptides did not visibly alter the microtubules in HeLa cells (Fig. 6D). However, a significant depolymerization of microtubules occurred when the cells were treated with these peptides in combination with 100 nM nocodazole (Fig. 6D). Western blotting was done to quantify the amount of polymer and soluble tubulin levels in Bim-treated cells (supplemental Fig. S5A). The polymer/soluble tubulin ratio was found to be 1.1 ± 0.1, 1.1 ± 0.07, 0.87 ± 0.1, and 0.56 ± 0.1 in control, Bim peptide, 100 nM nocodazole, and a combination of nocodazole- and Bim peptide-treated cells, respectively (supplemental Fig. S5B). Similarly, Western blotting was done to quantify the amount of polymer and soluble tubulin levels in Nos peptide-treated cells (supplemental Fig. S6A). The polymer/soluble ratio was found to be 1.1 ± 0.2, 0.99 ± 0.1, 0.86 ± 0.1, and 0.55 ± 0.1 in control, Nos peptide, 100 nM nocodazole, and a combination of nocodazole and Nos peptide-treated cells, respectively (Supplemental Fig. S6B).

The Depletion of LC8 Destabilizes Mitotic Spindle—The depletion of LC8 had a strong effect on the mitotic microtubules (Fig. 7A). The mitotic microtubules were depolymerized, and the affected cells formed aberrant mitotic spindles. Approximately 40% of the total mitotic cells produced disorganized spindles after LC8 depletion, suggesting that the protein...
is required for the stability of mitotic microtubules (Fig. 7A). The spindle disruption could also be due to abnormal dynein and myosin V functions in the cell. Furthermore, the depletion of LC8 also caused a substantial increase in the mitotic index in HeLa cells (Fig. 7B).

We also examined whether LC8-mediated microtubule stabilization plays a role in microtubule-kinetochore attachment by examining the status of Mad2, a checkpoint protein, in the mitotic cells. Mad2 is known to monitor microtubule-kinetochore attachment and to localize at the unattached kinetochores (48–50). Control HeLa cells showed a negligible amount of Mad2 at the kinetochores, whereas the LC8-depleted metaphase cells showed a much higher accumulation of Mad2 than that of the control cells. The positions of kinetochores are shown by Hec1, an outer kinetochore protein (51, 52) (Fig. 7C). The results indicated that the depletion of LC8 might perturb microtubule assembly dynamics and increased the accumulation of Mad2 at the kinetochores.

A Model Describing the Interaction between LC8 and Tubulin—

To understand the underlying mechanism of the stabilization of microtubules by LC8, we investigated the interactions between LC8 and tubulin dimers/polymers at the atomic scale in silico. The LC8 dimer (PDB code 1F96) structure was docked separately on to the tubulin dimer (PDB code 1JFF) and tubulin protofilaments (PDB code 3HKC), and the interactions were investigated among them in the energy-minimized LC8-tubulin complex models (supplemental Table S1). The predicted LC8 dimer-α/β-tubulin complex model has a calculated free energy (ΔG) of −5144 KJ/mol, and the LC8 dimer was predicted to dock at the junction of α and β tubulin (Fig. 8, A−C) predominantly through hydrogen bonds and charge interactions (supplemental Tables S1 and S2). Interestingly, the LC8...
occupied the groove on the tubulin dimer, which was at 180° from the kinesin/dynein-head interacting groove. Therefore, LC8 is predicted to localize toward the lumen of the microtubule. A meticulous analysis of the interactions suggested that similar residues of chain A and B of LC8 dimer (e.g. Tyr-32–His-55) interact with the β and α tubulin, respectively. In addition, the similar residues of chain A and B of LC8 dimer (e.g. Ser-64–Gly-89) interact with the α and β tubulin, respectively (supplemental Table S3). Most interestingly, the chain A of LC8 interacts with H2 helix, S3 sheets, and H2–S3 loop of α-tubulin, and the chain B of LC8 interacts with H7 helix of β-tubulin. Similarly, the LC8-chain A interacts with H1 and H3 helices of α-tubulin, and the LC8-chain B interacts with S8 and H10 helix of β-tubulin (Fig. 8B and supplemental Table S3). These fragments of α and β tubulin are responsible for lateral contacts/interactions of the tubulin protofilaments (53).

Furthermore, to understand the interactions between LC8 and microtubule protofilaments, we docked LC8 dimer (PDB code 1F96) on to the α/βα-tubulin trimers (PDB code 3HKC) and found that LC8 only binds to α and β tubulin junction but not to the β and α tubulin junction (Fig. 8D). These suggest that LC8 might bind to a microtubule with 8-nm periodicity along its length. Therefore, both models indicated multiple contacts between LC8 and tubulin that are spread over lateral interacting regions in the α/β-tubulin and LC8 might play a role in stabilizing the microtubule by stabilizing the inter-protofilament interactions (Fig. 8E, supplemental Table S2). Recently, several uncharacterized small structures that are periodically present along the length of the microtubule are found in the outer lumen of microtubules present in the axoneme and basal body (54, 55). Therefore, we speculate that LC8 could be present in some of the uncharacterized structures of the axoneme/basal body.

The dimeric form of LC8 efficiently binds to its partners, such as Bim, neuronal nitric-oxide synthase (21, 31, 32). Phosphorylation at serine 88 is known to destabilize LC8 homodimers due to steric hindrance at the dimeric interface (29). S88E (phosphomimetic mutant) LC8 monomers dimerize much less than the wild type LC8; thus, the mutant LC8 fails to interact with its binding partners, such as Bim (29). In S88E-LC8 mutant cells, LC8 exists in both monomer and dimer forms. Therefore, to predict the interaction between phosphorylated LC8 and α/β tubulin, we investigated the interactions in the probable complexes of S88E-LC8 dimer, LC8 monomer, and S88E-LC8 monomer with α/β-tubulin, independently. Interestingly, the stabilization energy (ΔSE) values of S88E-LC8-dimer–α/β-tubulin and LC8-monomer–α/β-tubulin complexes were found to be drastically reduced in comparison to the ΔSE value of LC8-dimer–α/β-tubulin. The topologies of the LC8 in the α/β-tubulin–LC8 complex changed with the changes in dimerization states of the LC8 (supplemental Fig. S7). A favorable electrostatic surface in the dimeric LC8 that is perhaps not preserved in its monomeric form could facilitate the interactions between LC8 dimer and α/β tubulin. The surface charge distribution of LC8 dimer is visibly different from that of the S88E-LC8, and this could affect the ΔSE in S88E-LC8-dimer–α/β-tubulin complex. In addition, topology of the S88E-LC8-dimer in the LC8-tubulin complex was quite different. These analyses suggested only LC8 dimers can bind to α/β-tubulin, and the phosphorylation of LC8 is likely to destabilize the LC8-tubulin interactions.

The models, however, do not explain how LC8 could bundle microtubules, which was observed both in vitro and in vivo experiments. Therefore, we speculate that LC8 may have multiple binding sites on tubulin, and some of them are solvent-exposed. However, binding to the highest affinity sites, which are likely to be buried inside the tube, might help to stabilize the filaments. The proposed model only suggests the likely positions of the highest affinity sites on the tubulin, and further studies will be needed to confirm these predictions.
DISCUSSION

In this study LC8 was found to interact directly with purified tubulin, to enhance tubulin assembly, and to stabilize microtubules in vitro and in cultured cells. Similar to well known structural microtubule-associated proteins such MAP2 and Tau, LC8 was found to decorate reconstituted microtubules in vitro. In HeLa cells, the overexpression of LC8 caused bundling of interphase microtubules, and microtubule bundles were mostly observed at places where LC8 was localized, ascertaining the interaction of LC8 with microtubules and its role in microtubule stability. Furthermore, molecular docking studies indicated that LC8 binds at the interface of structural microtubule-associated proteins such MAP2 and Tau, to enhance tubulin assembly, and to stabilize microtubules. Furthermore, molecular docking studies indicated that LC8 binds at the interface of αβ tubulin in dimeric form and helps to stabilize the protofilaments.

LC8 bound to brain microtubules with a dissociation constant of 2.6 ± 0.3 μM. Tau, a neuronal microtubule-associated protein, has been shown to bind to microtubules with a dissociation constant ranging from 16 to 900 nM (56, 57). The dissociation constant for two higher molecular weight Tau isoforms was found to be around 0.45 μM, whereas for four smaller isoforms the dissociation constant was found to be 0.9 μM (56). The bacterially expressed microtubule binding region of MAP2 binds to microtubules with a dissociation constant of 1.1 μM (58). The results suggested that the binding affinity of LC8 with microtubules is weaker than that of Tau and MAP2.

LC8 has been reported to interact with various proteins like Bim, neuronal nitric-oxide synthase, Drosophila RNA localization protein swallow, myosin V, neuronal scaffolding protein GSK, and IkBα (21, 31, 29, 59–61) in the dimeric state. In this study LC8 and S88E-LC8 (phosphomimetic mutant) were overexpressed in HeLa cells. LC8 can form functional dimers; however, S88E-LC8 is known to exist in the monomeric state (29). Although the overexpression of LC8 increased microtubule bundling and stability in HeLa cells, the overexpression of S88E-LC8 neither induced bundling nor increased the stability of microtubules. It might be possible that LC8 interacts with microtubules in the dimeric state and not in the monomeric state, as no change in microtubule network was observed in the presence of S88E-LC8 overexpression. Bim and Nos peptides have been reported to interact with LC8 dimers and to engage them (32). In the presence of Bim and Nos peptides, microtubules were found to be more susceptible to a low concentration of nocodazole, indicating that the peptides inhibit the interaction of LC8 with microtubules. The docking analysis indicated that the phosphorylation of LC8 reduced its ability to form dimers and consequently decreased its ability to interact with the αβ tubulin, supporting the finding that the overexpression of S88E-LC8 had no detectable effect on the microtubules in HeLa cells.

The depletion of LC8 had no apparent effect on the organization of interphase microtubules; however, when LC8 siRNA was used in combination with low concentration of nocodazole (100 nM), a significant depolymerization of interphase microtubules was observed as compared with the microtubules of the cells treated with only nocodazole. The results suggested that the depletion of LC8 made the microtubules more labile to a microtubule-depolymerizing agent. The effect of LC8 depletion in mitotic cells was more pronounced, as its depletion led to defective mitotic microtubules. In many cells the bipolar orientation was lost, leading to the formation of disorientated mitotic microtubules. Because mitotic microtubules are more labile than the interphase microtubules, they were more susceptible toward LC8 depletion. In addition Bim and nNOS peptides were found to inhibit the interaction of LC8 and microtubules. Bim and nNOS peptides alone did not cause the depolymerization of interphase microtubules but in combination with 100 nM nocodazole showed a significant depolymerization of microtubules as was observed when LC8 siRNA was used in combination with 100 nM nocodazole. The results of LC8 inhibition suggested that LC8 may function as a stabilizing factor for both the mitotic and interphase microtubules in cells.

The motors like kinesin-5 and kinesin-14 and the non-motor microtubule-associated proteins of the Ase1p family like PRC1 are known to cross-link the microtubules into bundles (62). We found that the overexpression of LC8 induced bundling of interphase microtubules; however, it did not affect mitotic spindle organization as reported with PRC1 (40). Previous studies showed that the overexpression of Ase1p members induced microtubule bundling and cross-linked mitotic microtubules, thereby stabilizing them (40, 63–67). The deleterious consequences of LC8 depletion on mitotic spindle microtubule organization indicated that LC8 might be involved in stabilizing the mitotic microtubules by cross-linking them. On the basis of in vitro and cellular studies, we suggest that the decoration of LC8 on microtubules might enhance the lateral interaction between the microtubules, leading to the bundling and stability of microtubules.

LC8 may cross-link microtubules leading to an enhanced stability of the microtubules. Stabilized microtubules may act as continuous tracks for motor proteins carrying the cargo and help them in efficient transport of the cargos. The bipolar spindle assembly was perturbed when LC8 was depleted, suggesting that LC8 plays a role in the mitotic spindle assembly and that it may have a role in the stabilization of the mitotic microtubules. Various proteins like asep1 have been shown to be important for the construction of the mitotic spindles, as they assist kinesins like kinesin-5 and kinesin-6 in cross-linking the microtubules (62). Likewise LC8 may also act as a microtubule-associated protein functioning in coordination with various spindle assembly proteins for the formation of proper chromosomal bipolar orientation and segregation.

Acknowledgments—We thank B. Jindal, S. Kapoor, and A. Rai for critical reading of the manuscript, Dr. A. Ghosh-Roy for the pET-LC8 clone, and Dr. S. Mazumdar and D. DattaGupta of Department of Chemical Sciences, Tata Institute of Fundamental Research for providing the Bim and nNOS peptides.

REFERENCES
1. Cheeseman, I. M., and Desai, A. (2008) Molecular architecture of the kinetochore-microtubule interface. Nat. Rev. Mol. Cell Biol. 9, 33–46
2. Kaverina, I., and Straube, A. (2011) Regulation of cell migration by dynamic microtubules. Semin. Cell Dev. Biol. 22, 968–974
3. Müsch, A. (2004) Microtubule organization and function in epithelial cells. Traffic 5, 1–9
4. Dent, E. W., and Gertler, F. B. (2003) Cytoskeletal dynamics and transport

LC8 Stabilizes Microtubules
in growth cone motility and axon guidance. *Neuron* 40, 209–227
5. Witte, H., and Bradke, F. (2008) The role of the cytoskeleton during neuronal polarization. *Curr. Opin. Neurobiol.* 18, 479–487
6. Schliwa, M., and Woehlke, G. (2003) Molecular motors. *Nature* 422, 759–765
7. Gadde, S., and Heald, R. (2004) Mechanisms and molecules of the mitotic spindle. *Curr. Biol.* 14, R797–R805
8. Panda, D., Samuel, J. C., Massie, M., Feinstein, S. C., and Wilson, L. (2003) Differential regulation of microtubule dynamics by three- and four-repeat Tau. Implications for the onset of neurodegenerative disease. *Proc. Natl. Acad. Sci. U.S.A.* 100, 9548–9553
9. Cowan, C. M., Bossing, T., Page, A., Shepherd, D., and Mudher, A. (2010) Soluble hyper-phosphorylated Tau causes microtubule breakdown and functionally compromises normal Tau in vivo. *Acta Neuropathol.* 120, 593–604
10. Lippens, G., Sillen, A., Landrieu, I., Amniai, L., Sibille, N., Barbier, P., Leroy, A., Hanoule, X., and Wieruszewski, I. M. (2007) Tau aggregation in Alzheimer’s disease. What role for phosphorylation? *Prion* 1, 21–25
11. Hummel, T., Krukkert, K., Roos, J., Davis, G., and Klämbt, C. (2000) Dynein light chain LC8 Stabilizes Microtubules
12. Graf, E. R., Heerssen, H. M., Wright, C. M., Davis, G. W., and DiAntonio, D., Sahin, A. A., den Hollander, P., and Kumar, R. (2004) Dynein light chain destabilizes a portion of the myosin’s coiled-coil domain.
13. Akhmanova, A., and Steinmetz, M. O. (2008) Tracking the ends. A dynein/dynactin molecular junction. *J. Neurosci.* 31, 15026–15034
14. Asakawa, K., Toya, M., Sato, M., Kanai, M., Kume, K., Goshima, T., Garcia, M. A., Hirata, D., and Toda, T. (2005) Mal3, the fission yeast EB1 homologue, cooperates with Bub1 spindle checkpoint to prevent monopolistic attachment. *EMBO Rep.* 6, 1194–1200
15. Goldstone, S., Reyes, C., Gay, G., Courthéoux, T., Dubarry, M., Tournier, S., and Gachet, Y. (2010) Tip1/CLIP-170 protein is required for correct chromosome poleward movement in fission yeast. *PLoS One* 5, e16034
16. Glotzer, M. (2009) Cytokinesis. GAP gap. *Curr. Biol.* 19, R162–R165
17. Ruthinasmv, K., and Panda, D. (2008) Kinetic stabilization of microtubule dynamic instability by benomyl increases the nuclear transport of p53. *Biochem. Pharmacol.* 76, 1669–1680
18. Paschal, B. M., and Vallee, R. B. (1987) Retrograde transport by the microtubule-associated protein MAP 1C. *Nature* 330, 181–183
19. King, S. M., and Patel-King, R. S. (1995) The solution structure of the pH-induced monomer of dynein light chain LC8 from *Drosophila* Futisch/22C10 is a MAP1B-like protein required for dendritic and axonal development.
20. Wagner, W., Fodor, E., Ginsburg, A., and Hammer, J. A., 3rd (2006) The molecular basis for neuronal nitric-oxide synthase.
21. Jaffrey, S. R., and Snyder, S. H. (1996) PIN. An associated protein inhibitor of neuronal nitric-oxide synthase.
22. Hummel, T., Krukkert, K., Roos, J., Davis, G., and Klämbt, C. (2000) PRC1 is a microtubule binding and bundling protein
23. Krukkert, K., Roos, J., Davis, G., and Klämbt, C. (2000) Dynein light chain phosphorylation controls macropinocytosis. *J. Biol. Chem.* 280, 654–659
24. Fan, J., Zhang, Q., Tochio, H., Li, M., and Zhang, M. (2001) Structural basis of neuronal nitric-oxide synthase. *Nat. Rev. Mol. Cell Biol.* 2, 9548–29035
25. Vadlamudi, R. K., Bagheri-Yarmand, R., Yang, Z., Balasenthil, S., Nguyen, H., and Nguyen, M. (2005) Dynein light chain 1 stabilizes a portion of the myosin’s coiled-coil domain. *Proc. Natl. Acad. Sci. U.S.A.* 107, 3493–3498
26. Yang, Z., Vadlamudi R. K., and Kumar, R. (2003) Dynein light chain for nuclear migration and septum positioning is temperature dependent in Aspergillus nidulans. *Mol. Microbiol.* 47, 291–301
27. Nakrafuro-Lerida, I., Martinez Moreno, M., Roncal, F., Gavilanes, F., Albar, J. P., and Rodríguez-Creso, I. (2004) Proteomic identification of brain proteins that interact with dynein light chain LC8. *Proteomics* 4, 339–346
28. Suzuki, K., and Panda, D. (2002) Perturbation of microtubule polymerization by quercetin through tubulin binding. A novel mechanism of its anti-proliferative activity. *Biochemistry* 41, 13029–13038
29. Yamada, R., and Panda, D. (2008) Microwave-induced polymerization of tubulin. Characteristics of the reaction and application to the large-scale purification of tubulin. *Arch. Biochem. Biophys.* 209, 29–40
30. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254
31. Mollinari, C., Kleman, J. P., Jiang, W., Schoen, H., Hunter, T., and Maragos, R. L. (2002) PRC1 is a microtubule binding and bundling protein essential to maintain the mitotic spindle midzone. *J. Cell Biol.* 157, 1175–1186
32. Mohan, R., and Panda, D. (2008) Kinetic stabilization of microtubule dynamics by estramustine is associated with tubulin acetylation, spindle abnormalities, and mitotic arrest. *Cancer Res.* 68, 6181–6189
33. Vakser, I. A., Matar, O. G., and Lam, C. F. (1999) A systematic study of low resolution recognition in protein–protein complexes. *Proc. Natl. Acad. Sci. U.S.A.* 96, 8477–8482
34. Hekkelman, M. L., Te Beek, T. A., Pettifer, S. R., Thorne, D., Attwood, T. K., and Vriend, G. (2010) Wiw. A protein structure bioinformatics Web service collection. *Nucleic Acids Res.* 38, W719–W723
35. Sobolev, V., Sorokine, A., Pintusky, J., Abola, E. J., and Edelman, M. (1999) Automated analysis of interatomic contacts in proteins. *Bioinformatics* 15, 327–332
36. Yvon, A. M., Wadsworth, P., and Jordan, M. A. (1999) Taxol suppresses dynamics of individual microtubules in living human tumor cells. *Mol. Biol. Cell* 10, 947–959
37. Mohan, P. M., Barve, M., Chatterjee, A., and Hosur, R. V. (2006) pH driven conformational dynamics and dimer-to-monomer transition in DCL8. *Protein Sci.* 15, 335–342
38. Piperno, G., LeDizet, M., and Chang, X. I. (1987) Microtubules containing acetylated α-tubulin in mammalian cells in culture. *J. Cell Biol.* 104, 289–302
39. Howell, B. J., Hoffman, D. B., Fang, G., Murray, A. W., and Salmon, E. D. (2000) Visualization of Mad2 dynamics at kinetochores, along spindle fibers, and at spindle poles in living cells. *J. Cell Biol.* 150, 1233–1250
40. Mayer, C., Filipei, I., Batac, J., Alford, L., and Paluh, J. L. (2006) An extended anaphase signaling pathway for Mad2p includes microtubule-organizing center proteins and multiple motor-dependent transitions. *Cell"
50. Zhang, D., Yin, S., Jiang, M. X., Ma, W., Hou, Y., Liang, C. G., Yu, L. Z., Wang, W. H., and Sun, Q. Y. (2007) Cytoplasmic dynein participates in meiotic checkpoint inactivation in mouse oocytes by transporting cytoplasmic mitotic arrest-deficient (Mad) proteins from kinetochores to spindle poles. *Reproduction* **133**, 685–695

51. DeLuca, J. G., Dong, Y., Hergert, P., Strauss, J., Hickey, J. M., Salmon, E. D., and McEwen, B. F. (2005) Hecl and nuf2 are core components of the kinetochore outer plate essential for organizing microtubule attachment sites. *Mol. Biol. Cell* **16**, 519–531

52. Diaz-Rodríguez E., Sotillo, R., Schvartzman, J. M., and Benezra, R. (2008) Hec1 overexpression hyperactivates the mitotic checkpoint and induces tumor formation in vivo. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 16719–16724

53. Nogales, E., Whittaker, M., Milligan, R. A., and Downing, K. H. (1999) High resolution model of the microtubule. *Cell* **96**, 79–88

54. Nicastro, D., Fu, X., Heuser, T., Tso, A., Porter, M. E., and Linck, R. W. (2011) Cryo-electron tomography reveals conserved features of doublet microtubules in flagella. *Proc. Natl. Acad. Sci. U.S.A.* **108**, E845–E853

55. Li, S., Fernandez, J. J., Marshall, W. F., and Agard, D. A. (2012) Three-dimensional structure of basal body revealed by electron cryo-tomography. *EMBO J.* **31**, 552–562

56. Makrides, V., Massie, M. R., Feinstein, S. C., and Lew, J. (2004) Evidence for two distinct binding sites for Tau on microtubules. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 6746–6751

57. Butner, K. A., and Kirschner, M. W. (1991) Tau protein binds to microtubules through a flexible array of distributed weak sites. *J. Cell Biol.* **115**, 717–730

58. Coffey, R. L., and Purich, D. L. (1995) Non-cooperative binding of the MAP-2 microtubule binding region to microtubules. *J. Biol. Chem.* **270**, 1035–1040

59. Crépieux, P., Kwon, H., Leclerc, N., Spencer, W., Richard, S., Lin, R., and Hiscott, J. (1997) IeBea physically interacts with a cytoskeleton-associated protein through its signal response domain. *Mol. Cell. Biol.* **17**, 7375–7385

60. Schnorrer, F., Bohmann, K., and Nüsslein-Volhard, C. (2000) The molecular motor dynein is involved in targeting swallow and bicoid RNA to the anterior pole of Drosophila oocytes. *Nat. Cell Biol.* **2**, 185–190

61. Naisbit, S., Valtschanoff, J., Allison, D. W., Sala, C., Kim, E., Craig, A. M., Weinberg, R. J., and Sheng, M. (2000) Interaction of the postsynaptic density-95/guanylate kinase domain-associated protein complex with a light chain of myosin-V and dynein. *J. Neurosci.* **20**, 4524–4534

62. Peterman, E. J., and Scholey, J. M. (2009) Mitotic microtubule cross-linkers. Insights from mechanistic studies. *Curr. Biol.* **19**, R1089–R1094

63. Pellman, D., Bagget, M., Tu, Y. H., and Fink, G. R. (1995) Microtubule-associated proteins required for anaphase spindle movement in *Saccharomyces cerevisiae*. *J. Cell Biol.* **130**, 1373–1385

64. Verni, F., Somma, M. P., Gunsalus, K. C., Bonaccorsi, S., Belloni, G., Goldberg, M. L., and Gatti, M. (2004) Feo, the *Drosophila* homolog of PRC1, is required for central-spindle formation and cytokinesis. *Curr. Biol.* **14**, 1569–1575

65. Loïodice, I., STaub, J., Setty, T. G., Nguyen, N. P., Paoletti, A., and Tran, P. T. (2007) Ase1p organizes antiparallel microtubule arrays during interphase and mitosis in fission yeast. *Mol. Biol. Cell* **16**, 1756–1768

66. Schuyler, S. C., Liu, J. Y., and Pellman, D. (2003) The molecular function of Ase1p. Evidence for a MAP-dependent midzone-specific spindle matrix. *J. Cell Biol.* **160**, 517–528

67. Janson, M. E., Loughlin, R., Loïodice, I., Fu, C., Brunner, D., Nédélec, F. J., and Tran, P. T. (2007) Cross-linkers and motors organize dynamic microtubules to form stable bipolar arrays in fission yeast. *Cell* **128**, 357–368