INTRODUCTION

Microtubules (MTs) are major components of the cytoskeleton involved in fundamental cellular processes. They are rigid, polar, and dynamical elements that polymerize and depolymerize at both ends. The stochastic switching between growing and shortening phases is termed dynamic instability (Mitchison and Kirschner, 1984). In living cells, they form arrays that either radiate from structured MT-organizing centers such as the centrosome or are self-organized into linear bundles that assume different configurations, depending on the cell type (Bartolini and Gundersen, 2006). Within these arrays, MTs can be straight or highly curved (deformed MTs), presumably depending on the functions in which they are engaged (Rusan and Wadsworth, 2005; Brangwynne et al., 2006; Wasteneys and Ambrose, 2009). An example of MT deformations in cells includes the formation of MT bundles. Indeed, to create bundles, physical collisions between MTs at angles up to 40° might result in bending deformations so that they coalign (Shaw et al., 2003; Dixit and Cyr, 2004). Furthermore, beside individual MTs, small groups of MTs such as bundles also assume deformations. In plant cells that display box-like geometry, with sharp edges marking the boundaries between adjacent faces, MT bundles wind around the cell cortex and thus are deformed to adapt their organization to these physical constraints (Wasteneys and Ambrose, 2009; Ambrose et al., 2011). In fission yeast, MT bundle deformations have also been observed in correlation with nucleus positioning (Tran et al., 2001). These observations suggest that in living cells, the mechanical behavior of MTs/MT bundles is variable and must be locally regulated, depending on the functions in which they are engaged. Because MTs are highly rigid structures, with a persistence length ($L_p$) of 1–8 mm for naked single MTs (Hawkins et al., 2010), one essential question to understand is how they can be deformed at a scale of a few micrometers. In vivo, MT $L_p$ was estimated to be $\sim 30 \mu m$ in animal culture cells (Gardel et al., 2008). In these cells, MT bending was suggested to be due to forces exerted by actin filaments and myosin motors or MT molecular motors (Brangwynne et al., 2006; Gardel et al., 2008; Bicek et al., 2010).

ABSTRACT

Microtubules (MTs) are dynamic cytoskeletal elements involved in numerous cellular processes. Although they are highly rigid polymers with a persistence length of 1–8 mm, they may exhibit a curved shape at a scale of few micrometers within cells, depending on their biological functions. However, how MT flexural rigidity in cells is regulated remains poorly understood. Here we ask whether MT-associated proteins (MAPs) could locally control the mechanical properties of MTs. We show that two major cross-linkers of the conserved MAP65/PRC1/Ase1 family drastically decrease MT rigidity. Their MT-binding domain mediates this effect. Remarkably, the softening effect of MAP65 observed on single MTs is maintained when MTs are cross-linked. By reconstituting physical collisions between growing MTs/MT bundles, we further show that the decrease in MT stiffness induced by MAP65 proteins is responsible for the sharp bending deformations observed in cells when they coalign at a steep angle to create bundles. Taken together, these data provide new insights into how MAP65, by modifying MT mechanical properties, may regulate the formation of complex MT arrays.

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Abbreviations used: Lp, persistence length; MAP, microtubule-associated proteins; MT, microtubule.

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Microtubule rigidity decreases by MAPs

K2010). In the presence of 100 nM MAP65-1 or Ase1 (with apparent sense of MAPs have been suggested to alter MT flexibility. Of identified MAPs, only two have been reported to modify MT flexural rigidity in vitro, namely the neuronal MAPs tau and MAP2, which stiffen them (Mickey and Howard, 1995; Felgner et al., 1997). In contrast, increased flexibility of MTs due to MAPs has not been described. In this study, to explore whether MT deformations observed in living cells could be due to MAPs, we focus on MT cross-linkers since, as discussed, MT deformations might be a key process in generating MT bundles in eukaryotic cells (Dixit and Cyr, 2004; Bartolini and Gundersen, 2006; Bratman and Chang, 2008).

Beside molecular motors, the major MAPs that cross-link MTs in cells are members of the conserved MAP65 family (Mollinari et al., 2002; Schuyler et al., 2003; Smertenko et al., 2004; Stoppin-Mellet et al., 2013). Gene analysis reveals nine isoforms in the plant model Arabidopsis thaliana (MAP65-1 to -9; Hussey et al., 2002) and one each in vertebrates (PRC1; Mollinari et al., 2002) and yeast (Ase1; Schuyler et al., 2003). Ase1/MAP65-1 are associated with the cortical MTs during interphase (Schuyler et al., 2003; Lucas et al., 2011), and Ase1/PRC1/MAP65-1 play key roles in organizing antiparallel MTs at the midzone during mitosis (Mollinari et al., 2002; Smertenko et al., 2004; Janson et al., 2007; Gaillard et al., 2008). To explore whether MAP65 modify MT mechanical properties, we developed an original assay based on an MT micropattemning method (Portran et al., 2013) and hydrodynamic flux (Venier et al., 1994). We demonstrate that MAP65-1/Ase1 drastically increase the flexibility of both single and cross-linked MTs. This effect is independent of MT dynamical changes and is directly mediated by the MAP65 MT-binding domain. To determine whether the softening effect of MAP65 on MTs was determinant in creating bundles, we reconstituted physical collisions between growing MTs/MT bundles and found that the decrease in MT flexural rigidity mediated by MAP65/Asel is required to enable the bending and coagiment of MTs at steep angles. Taken together, these data provide new insights into how these key proteins, by modifying MT mechanical properties, control MT–MT encounters and result in the formation of complex MT bundle arrays. More generally, they suggest that MT softening by MAPs may represent an original mechanism that could regulate the plasticity of MT networks in cells.

RESULTS

MAP65-1/Ase1 increase the flexibility of individual MTs

To evaluate the effect of MAP65-1 and Ase1 on MT bending stiffness, we measured Lp for MTs growing in the presence of these two MAP65s. We set up an assay in which fluorescent MTs elongate from seeds immobilized on a bar-shaped micropatterned surface (Portran et al., 2013) and are bent by hydrodynamic flow applied perpendicular to the growing MTs (Figure 1A and Supplemental Movies S1 and S2). To measure MT rigidity from flow-induced MT deformations, we adapted the equations for elastic filaments (Berro et al., 2007) and determined the MT Lp using the best-fit solution to the MT configuration (Supplemental Methods). MTs growing in the absence of MAPs have Lp = 1.58 ± 0.62 mm (Table 1 and Figure 1B), which is consistent with previous reported values (Hawkins et al., 2010). In the presence of 100 nM MAP65-1 or Ase1 (with apparent Kd of, respectively, 21 ± 2 and 41 ± 7 nM; Supplemental Figure S1, A and B), MT Lp decreases by a factor of 4 and 3.2, respectively (Table 1, Figure 1B, and Supplemental Movie S2). This effect is concentration dependent and is already observed at 1 nM MAP65-1 (Figure 1C).

To investigate whether the increase in MT flexibility is unique to MAP65-1 and Ase1, we next determined the effect of another member of the plant MAP65 family on MT Lp. Indeed the plant MAP65, in contrast to the case for mammals and yeast, contains several members (Hussey et al., 2002). Among them, we chose MAP65-4, which associates with kinetochore fibers during mitosis and organizes MTs in bundles in vitro but differently than MAP65-1: MAP65-4 is oriented perpendicular to the MT lattice (vs. oblique for MAP65-1) and coaligns MTs with 15-nm MT interspacing (vs. 30 nm for MAP65-1; Gaillard et al., 2008; Fache et al., 2010). In the presence of 100 nM MAP65-4 (apparent Kd of 19 ± 2 nM; Supplemental Figure S1C), MT Lp = 1.39 ± 0.47 mm, which is not significantly different from that for MTs in the absence of MAPs (Table 1 and Figure 2, B and C).

MAP65 MT-binding domain is required to modulate MT stiffness

We next examined the relative contributions of the functional domains of MAP65-1 to increased MT flexibility. Indeed, MAP65-1, like other MAP65-family proteins, exhibits a C-terminal MT-binding domain and an N-terminal coiled-coil region (called the projection domain) required for its homodimerization (Figure 2A). The MT-binding domain is responsible for the preferential antiparallel orientation of the bundled MTs, whereas the projection domain length determines MT interspacing (Gaillard et al., 2008; Tulin et al., 2012). In contrast to Ase1 and PRC1, MAP65-1 binds to MTs as a monomer and further dimerizes when it encounters another MAP65-1 bound to an adjacent MT (Gaillard et al., 2008; Tulin et al., 2012). First, we generated a fragment carrying the MT-binding domain of MAP65-1 (named MAP65-1(MBD); Figure 2A). At a concentration of 100 nM, MAP65-1(MBD) decreases the Lp of growing MTs (0.83 ± 0.34 mm vs. 1.58 ± 0.62 nm without MAP65) but with a lower efficiency than full-length MAP65-1 (Table 1 and Figure 2, B and C). This lower efficiency could be related to its lower MT-binding affinity, which is 100-fold weaker than that of full-length MAP65-1 (apparent Kd = 2.1 ± 4 μM; Supplemental Figure S1D). The lower Kd value of the MT-binding domain compared with that of the full-length MAP65-1 might result from 1) incorrect folding due to the absence of the projection domain and/or 2) its binding to MTs as a monomer, in contrast to full-length MAP65-1, which binds bundled MTs as a dimer.

We next generated chimeras with the MT-binding domain of MAP65-1 and the dimerization domain of MAP65-4 and vice versa (referred to as Chimeras 4-1 and Chimera 1-4, respectively; Figure 2A). Chimera 4-1 hardly bundled MTs and thus could not be tested in this study, whereas Chimera 1-4 had an affinity for MTs similar to that of MAP65-1 and MAP65-4 (Supplemental Figure S1E). In the presence of 100 nM Chimera 1-4, MT Lp = 1.29 ± 2.42 mm, a value not significantly different from the one in the absence of MAPs (Table 1 and Figure 2, B and C). Thus Chimera 1-4, like MAP65-4, had no effect on MT rigidity. These data show that the MT-binding domain of MAP65-1 mediates its MT-softening effect.

MAP65-1/Ase1 increase MT-bundle flexibility

To determine whether MAP65-1 and Ase1 also modulate the global mechanical properties of cross-linked MTs, we compared the Lp of growing MT bundles generated by MAP65-1, Ase1, MAP65-4, and Chimera 1-4 (Figure 3 and Supplemental Movies S3 and S4). We used the same experimental procedure as before but
FIGURE 1: MAP65-1/Ase1 decrease the flexural rigidity of individual MTs. (A) Measurement of MT persistence length. (a) Experimental setup. MT seeds are introduced in a flowthrough chamber composed of a micropattern slide (bar shape) saturated with NeutrAvidin and a glass support. They are aligned on functionalized bar patterns by the flow and attached on the micropattern surface via biotin–NeutrAvidin link (step 1). MT seeds are further elongated by the addition of Alexa-labeled tubulin in the presence or absence of MAP65 and in the presence of fluorescent beads (step 2). When MTs reach a length of 10 μm on average, the elongation mix is perfused into the flow chamber perpendicular to the elongating MTs in order to bend them (step 3). When the flow speed reaches its maximum and when it is stabilized, MT bending is measured (step 4). (b) Time series of bending MTs that elongate in the absence or presence of 100 nM MAP65-1 or Ase1. MTs are in green; MT seeds and beads are in red. (c) Superposition of the images in (b), showing the amplitude of the MT bending (red arrows). (B) Histograms of the ratio between the $L_p$ of single MTs grown in the absence of MAPs and the $L_p$ of MTs grown in the presence of 100 nM MAP65-1 or Ase1. MAP65-1 and Ase1 significantly decrease MT $L_p$. (C) Plot of the ratio of MT $L_p$ in the presence of different concentrations of MAP65-1 (1–100 nM).
cross-linked MTs was the same all along the bent, growing bundle. First, we focused on MAP65-4 and Chimera 1-4, which do not affect the mechanical properties of single MTs (Table 1). Bundles composed of two MTs had $L_p = 3.49 \pm 0.5$ (MAP65-4) and cross-linked MTs was the same all along the bent, growing bundle. First, we focused on MAP65-4 and Chimera 1-4, which do not affect the mechanical properties of single MTs (Table 1). Bundles composed of two MTs had $L_p = 3.49 \pm 0.5$ (MAP65-4) and $L_p = 1.04$ (Chimera 1-4). Immobilized MT seed bundles, instead of individual MT seeds, on the micropatterned surface (Figure 3A). To measure the bundle’s $L_p$, we considered only bundles having a maximum of three MTs, because with more, we could not ascertain that the number of MTs was the same all along the bent, growing bundle.

TABLE 1: MT persistence length in the presence of MAP65.

| Condition          | Number of MTs | MT length (μm) | $L_p$ (mm) | $L_p$ without MAP/ $L_p$ MAP65 |
|--------------------|---------------|---------------|------------|-------------------------------|
| Without MAP65      | 9             | 10.39 ± 0.65  | 1.58 ± 0.62| 1.00                          |
| Ase1               | 5             | 10.50 ± 0.80  | 0.45 ± 0.18| 3.22                          |
| MAP65-1            | 13            | 11.62 ± 1.17  | 0.39 ± 0.16| 4.05                          |
| MAP65-1(MBD)       | 4             | 8.30 ± 0.63   | 0.81 ± 0.34| 1.79                          |
| MAP65-4            | 4             | 13.81 ± 2.17  | 1.39 ± 0.47| 1.04                          |
| Chimera 1-4        | 6             | 13.00 ± 2.42  | 1.29 ± 0.65| 1.12                          |

Effective number, average length, and calculated $L_p$ for single, growing MTs in the absence or presence of 100 nM MAP65. Parameter values are reported with SD.

FIGURE 2: The MT-binding domain of MAP65-1 increases MT flexibility. (A) MAP65-1, MAP65-1(MBD), MAP65-4, and Chimera 1-4 constructs. MAP65-1 and MAP65-4 are divided into two domains: the projection and the MT-binding domain. The most conserved motif is underlined. Chimera 1-4 was obtained by replacing the projection domain of MAP65-4 with the projection domain of MAP65-1. (B) (a) Time series of bending MTs that elongate in the presence of 100 nM MAP65-4/Chimera 1-4/MAP65-1(MBD). MTs are in green; MT seeds and beads are in red. (b) Superposition of the images in (a) showing the amplitude of the bending (red arrows). (C) Histogram of the ratio between the $L_p$ of single MTs grown in the absence of MAPs and the $L_p$ of MTs grown in the presence of 100 nM MAP65-4, Chimera 1-4, and the MT-binding domain of MAP65-1.
2.57 ± 0.6 mm (Chimera1-4), respectively (Table 2). The \( L_p \) of bundles with three MTs further increases, to 4.16 (MAP65-4) and 4.07 mm (Chimera1-4; Table 2). In contrast, the rigidity of bundles assembled in the presence of MAP65-1 or Ase1 remains constant, as the number of MTs increases from two to three \( (L_p = 1.3 \text{ mm} \ [\text{two or three MTs}] \text{ for MAP65-1}; \ L_p = 1.05 \text{ [two MTs]} \text{ and } 1.16 \text{ mm} \ [\text{three MTs}] \text{ for Ase1}; \text{ Table 2}).\)

The \( L_p \) of bundles depends on three factors: 1) the orientation of the bundle with respect to the flow, 2) the MT arrangement in the bundle, and 3) the length of the link between MTs, as illustrated in Supplemental Figure S2, a–f. Because we could hardly extract the two first parameters from our experimental data, we computed the flexural rigidity \( (L_p \times k_B T) \), where \( k_B \) is the Boltzmann constant and \( T \) is the absolute temperature) of these different possible
bundle configurations (Supplemental Figure S2 and Supplemental Methods). First, we assumed that MTs are linearly arranged inside bundles with a relative orientation toward the flow as illustrated by configurations (a) and (c) in Supplemental Figure S2. In these bundles, the predicted stiffness scales linearly with the number of MTs (Supplemental Figure S2), in agreement with the \( L_p \) measured for bundles induced by MAP65-4 and Chimera 1-4 (Table 2). However, for bundles induced by MAP65-1 and Ase1, we measured a value of \( L_p \) that is constant and independent of the number of MTs in the bundle (Table 2). These data show that the softening effect of these two MAP65 on single MTs is maintained when they are bundled. Second, we turned to the other possible configurations for MTs in the bundles, for which one predicts larger stiffness (Supplemental Figure S2, b and d–f). Because we did not find a large variability in bundle \( L_p \), we conclude that these configurations are less likely to happen, indicating that configurations (a) and (c) are favored in our experimental setup.

**Encountering events**

To investigate whether the MT-softening effect of MAP65-1 and Ase1 is deterministic in creating bundles, we reconstituted physical collisions between single, growing MTs using a biomimetic system and total internal reflection fluorescence (TIRF) microscopy (Figure 4A and Supplemental Movie S5). We were able to mimic the MT collision events observed in living cells (Shaw et al., 2003; Dixit and Cyr, 2004), that is, crossover, touch-and-run, and bundling (Supplemental Figure S3 and Supplemental Movies S6–S8). The frequency of each of these outcomes in the presence of MAP65 was determined as a function of MT collision angles and MT polarity (Figure 4C and Supplemental Figure S3), with MT polarity being based on measurement of MT dynamics. In the presence of MAP65-1 and Ase1, the MT bundling was restricted to antiparallel MTs that encounter one another at angles up to 45°, in agreement with previous observations (Janson et al., 2007; Tulin et al., 2012) and result in a sharp bend of the incident MTs at the site of the collision (Figure 4, B and C, and Supplemental Figure S3). In contrast, in the presence of MAP65-4, MTs were not able to bundle MTs at collision angle >22° (Figure 4C). Below this value, bundling occurred whatever the polarity of MTs, in agreement with previous data demonstrating the nonselectivity of MAP65-4 for MT polarity (Fache et al., 2010). Finally, in the presence of Chimera 1-4, which does not modify MT \( L_p \), we observed MT bundling at angles up to 39° whatever the polarity of MTs (Figure 4C), whereas the bundling efficiency at angles >25° was low compare to that observed for MAP65-1 (Supplemental Figure S3D). Overall these results show that the ability of MAP65-1 to bundle MTs at steep angles requires an increase in MT flexibility directly controlled by its MT-binding domain.

**DISCUSSION**

In line with the softening effect of MAP65-1 and Ase1 on MT bundles (Figure 3), we reconstituted physical collisions between growing MT bundles in the presence of these proteins. In this assay, MTs elongate from MT seed bundles as described in Figure 3. The outcomes between growing MT bundles resulted in the same events as observed for individual MTs (Figure 4D and Supplemental Figure S4). In particular, MT bundles induced by MAP65-1 and Ase1 coaligned very efficiently at angles of up to 45° in order to generate a thicker bundle, whereas those induced by MAP65-4 and Chimera 1-4 were not able to coalign at angles >25° (Figure 4D and Supplemental Figure S4). Of interest, in the presence of MAP65-1, the steep angle collisions between bundles (>45°) were sometimes associated with a local deformation of the resident bundles over a length of 5 μM (Supplemental Figure S5). However, the occurrence of these events was too scarce to be reliably quantified. Conversely, such deformations were never observed in the presence of MAP65-4 or Chimera 1-4.

**TABLE 2: MT bundle persistence length in the presence of MAP65.**

| Condition | Number of bundles | Bundle length (µm) | \( L_p \) (mm) of bundles of two MTs | Number of bundles | Bundle length (µm) | \( L_p \) (mm) of bundles of three MTs |
|-----------|-------------------|-------------------|-------------------------------------|-------------------|-------------------|-------------------------------------|
| MAP65-4   | 6                 | 16 ± 3.97         | 3.49 ± 0.98                         | 3                 | 14.35 ± 1.06      | 4.16 ± 1.24                         |
| Chimera 1-4 | 9                 | 15.75 ± 2.93      | 2.58 ± 1.07                         | 7                 | 15.59 ± 3.68      | 4.07 ± 1.15                         |
| MAP65-1   | 2                 | 16.16 ± 3.95      | 1.31 ± 0.57                         | 7                 | 14.91 ± 1.3       | 1.30 ± 0.80                         |
| Ase1      | 13                | 14.94 ± 4.31      | 1.05 ± 0.44                         | 6                 | 16.59 ± 3.8       | 1.16 ± 0.66                         |

Effective number, average length, and calculated \( L_p \) for growing MT bundles in the presence of 100 nM MAP65. Bundles are composed of two or three MTs. Parameter values are reported with SD.
and consequently might not prevent MT sliding inside the bundle during their deformation. This suggests that either MAP65 diffuses between cross-linked MTs or MAP65 adopts different configurations. Indeed, recent studies show that Ase1, although decreasing MT sliding velocities generated by motors, does not inhibit it (Braun et al., 2011). Furthermore, Kapitein et al. (2008) showed that Ase1 diffuses between cross-linked MTs. In this study, we predicted that the bundle length should scale with the length of the MAP65 bonds (Supplemental Figure S2 and Supplemental Table S3), which is, respectively, 6 nm (Ase1; Schuyler et al., 2003), 15 nm (MAP65-4; Fache et al., 2010), and 30 nm (MAP65-1; Gaillard et al., 2008). However, our experimental data indicate a different picture. In particular, MAP65-1 and Ase1, which generate linearly along a single tubulin dimer (Subramanian et al., 2010). With this in mind, it is tempting to speculate that this domain, when bound to MTs, induces important structural changes within and/or between dimers that increase the freedom of interdimer motion during the bending of MTs. In contrast, the neuronal MAP tau binds MTs through multiple sites along but also across protofilaments (Santarella et al., 2004), suggesting that this MAP could increase MT rigidity by bridging protofilaments both laterally and longitudinally, thus limiting tubulin interdimer motions. These observations point out the importance of further study of the physical mechanisms underlying changes in MT lattice in the presence of MAPs.

Besides modifying the mechanical properties of single MTs, MAP65-1 and Ase1 also increase the flexibility of cross-linked MTs and consequently might not prevent MT sliding inside the bundle during their deformation. This suggests that either MAP65 diffuses between cross-linked MTs or MAP65 adopts different configurations. Indeed, recent studies show that Ase1, although decreasing MT sliding velocities generated by motors, does not inhibit it (Braun et al., 2011). Furthermore, Kapitein et al. (2008) showed that Ase1 diffuses between cross-linked MTs. In this study, we predicted that the bundle length should scale with the length of the MAP65 bonds (Supplemental Figure S2 and Supplemental Table S3), which is, respectively, 6 nm (Ase1; Schuyler et al., 2003), 15 nm (MAP65-4; Fache et al., 2010), and 30 nm (MAP65-1; Gaillard et al., 2008). However, our experimental data indicate a different picture. In particular, MAP65-1 and Ase1, which generate
very different MT-interspace lengths, induce bundles having comparable \( L_p \) values.

Our results reveal intrinsic biophysical properties of MAP65 that are likely important for a wide range of cellular processes. In particular, MT encounters at steep contact angles have been suggested to be sufficient to explain the proper self-organization of MTs into ordered cortical bundles in acientosomal plant cells (Allard et al., 2010; Eren et al., 2010). Because MAP65-1 and its homologue, MAP65-2, induce MT bundling in vivo (Lucas et al., 2011; Honukhse et al., 2012), we propose that these two MAP65, by softening and bundling MTs, are the key proteins involved in these processes. Although these mechanisms have been extensively observed in plant cells, we suggest that they may also apply to a wide range of differentiated eukaryotic cells that create self-ordered bundle arrays. Furthermore, the MT bundle softness due to MAP65-1 might facilitate their growth between adjacent cell faces to create the typical cortical arrays that bound different faces of the plant cells and be involved in regulating cell expansion. In line with our observations, recent in vivo studies from Honukhse et al. (2012) suggested that the transfacial MT bundles could be guided through MAP65-mediated CLASP localization. Beside MT cortical deformations in interphase, the regulation of MT mechanical properties by MAP65-1/Ase1 might also have implications for organizing the mitotic midzone. Indeed, to build the core of the midzone, which gives the spindle structural integrity, MAP65-1/Ase1/PRC1 cross-link antiparallel overlapping MTs that grow from the two opposite poles (Mollinari et al., 2002; Gaillard et al., 2008; Bieling et al., 2010; Subramanian et al., 2010; Portran et al., 2013). An increase in MT flexibility must allow them to coalign after encountering at steep angles (up to 40°), favoring the bundling of numerous MTs and subsequent midzone formation (Portran et al., 2013). More generally, MT buckling and bending are commonly observed in the cytoplasm of various eukaryotic cells (Brangwynne et al., 2006). However, the MT curvature that is observed at a scale of a few micrometers in cells is far from compatible with the MT \( L_p \) measured in vitro (1–8 nm; Bicek et al., 2009). In this study, by showing that MAPs significantly soften MTs in vitro, we suggest that this type of protein could be at the origin of MT deformations observed in the cytoplasm of various living cells and thus are able to regulate the local mechanical properties of MTs. Beside MAPs, other molecular mechanisms such as posttranslational modifications of tubulin could affect the MT lattice and thus alter MT flexural rigidity.

To conclude, MAP-induced MT flexibility might have numerous cell biological consequences, since it represents an alternative and simpler way for MT arrays to organize compared with assembly/disassembly processes. Hence an increase in MT flexibility should allow them to explore the cellular space laterally for binding partners, bend, and continue to grow in a new direction when encountering obstacles and/or coalign with other MTs. Thus MT softening by MAPs is a new mechanism to regulate the plasticity of MT networks in cells.

**MATERIALS AND METHODS**

**Protein purification**

Bovine brain tubulin was purified according to Vantard et al. (1994) in BRB80 buffer (80 mM 1,4-piperazinediethanesulfonic acid, pH 6.8, 1 mM ethylene glycol tetraacetic acid, and 1 mM MgCl\(_2\)). Fluorescent tubulin (Alexa 488–labeled tubulin and Alexa 568–labeled tubulin) and biotinylated tubulin were prepared according to Hyman et al. (1991). Recombinant MAP65-1/4, Chimera 1-4, and MAP65-1(MBD) were purified according to Gaillard et al. (2008), and Ase1 (kindly provided by Marcel Janson, Laboratory of Cell Biology, Wageningen, The Netherlands) according to Janson et al. (2007). Chimera 1-4 is a fusion of the projection domain of MAP65-1 (amino acids [aa] 1–340) and the MT-binding domain of MAP65-4 (aa 320–648).

**Determination of the apparent \( K_d \) of MAP65**

Taxol-stabilized MTs were prepared by incubating purified tubulin with equimolar concentrations of taxotere in BRB80 buffer supplemented with 1 mM GTP for 30 min at 37°C. Various concentrations of green fluorescent protein (GFP)–MAP65 (in MAP buffer: 50 mM NaPi, pH 7.9, 0.1 M NaCl, 0.5 mM dithiothreitol [DTT]) were mixed with 2 μM MTs in binding buffer (1× BRB80/0.5x MAP buffer). After 30 min at room temperature, samples were centrifuged during 20 min at 100,000 × g at 25°C. Supernatant was kept, and the pellet was resuspended in binding buffer supplemented with 0.5 M NaCl to detach MAP65 from MTs. The amount of GFP-MAP65 in the pellet and the supernatant was measured by spectrofluorimetry (excitation, 490 nm; emission, 512 nm). All tubes were silanized to limit nonspecific adsorption of MAP65 (Sambrook et al., 1989). Ase1 \( K_d \) was determined according to Portran et al. (2013).

**In vitro microtubule-encountering assays**

**Perfusion chambers**

Glass and coverslips were cleaned by successive chemical treatments as described (Portran et al., 2013). After exposure to air plasma (2 min at 60 W), coverslips were functionalized overnight in a solution of Silane-PEG-Biotin (3.5 kDa, 1 mg/ml in ethanol, 96.5%, plus 30 mM of HCl; SuSoS, Dübendorf, Switzerland) at room temperature. Glasses were passivated overnight in a solution of Silane-PEG (30 kDa, 1 mg/ml in ethanol, 96.5%, plus 30 mM HCl; Creative PEGworks, Winston Salem, NC) at room temperature. Glasses and coverslips were successively washed in ethanol and ultrapure water, dried with filtered air, and stored at 4°C away from dust. A flow cell chamber with an approximate volume of 20 μl was constructed with double-sided tape (70 μm height) between the glass and the coverslip. The perfusion chamber was coated with NeutrAvidin (25 μg/ml in 1% bovine serum albumin [BSA] in BRB80; Pierce, Rockford, IL), washed with 300 μl of 1% BSA in BRB80, and further passivated for 1 min with PLL-g-PEG (2 kDa, 0.1 mg/ml in 10 mM 4-(2-hydroxyethyl)-1-piperazinetanesulfonic acid, pH 7.4; Jenkem, Allen, TX).

**MT seed preparation**

MT seeds were obtained by polymerizing 20 μM tubulin (80% biotinylated tubulin and 20% Alexa 568–labeled tubulin) in the presence of 0.5 mM GMPCPP in BRB80 at 37°C for 1 h. Next, 2 μM taxotere was added, and MT seeds were further incubated at room temperature for 30 min. MT seeds were then centrifuged for 5 min at 300,000 × g and resuspended in an equal volume of BRB80 supplemented with 0.5 mM GMPCPP and 2 μM taxotere. They were stored in liquid nitrogen and quickly warmed at 37°C before use. Seed bundles were obtained by incubating 0.3 μM MT seeds with 0.1 μM MAP65-1, MAP65-4, Ase1, or Chimera 1-4 for 10 min at room temperature.

**In vitro encountering assays**

MT seeds were incubated for 5 min in the perfusion chamber. They were then elongated with an elongation mix containing 22 μM tubulin (30% Alexa 568–labeled tubulin and 70% unlabeled tubulin), 1 mM GTP, an oxygen scavenger cocktail (120 μg/ml glucose, 8 μg/ml catalase, and 40 μg/ml glucose oxidase), 20 μM DTT (Sigma-Aldrich, St. Louis, MO), 1% BSA, and 0.025% methyl cellulose (1500 CP; Sigma-Aldrich) in mix buffer (1× BRB80/0.5x MAP buffer) in the presence or the absence of MAP65. MT dynamics was visualized using an objective-based azimuthal ilas2 TIRF microscope (Nikon Eclipse Ti [Melville, NY], modified by Roper Scientific [Tucson, AZ]) and Evolve 512 camera (Photometrics,
Tucson, AZ). The microscope stage was kept at 32°C using a warm stage controller (MC60; Linkam, Tadworth, United Kingdom). Excitation was achieved using 491- and 561-nm lasers (Roper Scientific, France). Time-lapse recording (one frame every 5 s) was performed for 30 min using MetaMorph software (version 7.7.5; Molecular Devices, Sunnyvale, CA). Movies were processed to improve signal/noise ratio (equalize light, low pass, and flatten background filters of MetaMorph software). MT bundle elongation and dynamics were analyzed using kymographs generated by MetaMorph and analyzed with ImageJ (National Institutes of Health, Bethesda, MD). The statistical significance was determined using Student’s t test. The contact angle was measured between two MTs or two MT bundles preceding the behavior of encountering. The frequency for each behavior was calculated for nine contact angle ranges of 10° from 0 to 90°. MT polarity was determined by measuring MT growth rates, since MT minus ends grow at lower rates than MT plus ends.

Microtubule flexibility measurements

Methods and Supplemental Figure S6 for a detailed description. To extract MT position during bending experiments, we used the plug-in *JFilament* of ImageJ. To measure the fluid velocity we used the plug-in *MTtrackJ* of ImageJ. We collected the position of the beads and proceeded to the next image by connecting points associated with the same bead. We only took into account beads that were as bright as the beads, assuming that these beads were in the same z-position as MTs. Therefore the bead velocity was the same as the velocity of the fluid experienced by MTs. We calculated the average velocity of the flow for each frame. If beads could not be seen for a few frames (∼1–10), we interpolated the velocity between frames.

Image acquisition and velocity measurement. To extract MT position during bending experiments, we used the plug-in *JFilament* of ImageJ. To measure the fluid velocity we used the plug-in *MTtrackJ* of ImageJ. We collected the position of the beads and proceeded to the next image by connecting points associated with the same bead. We only took into account beads that were as bright as the beads, assuming that these beads were in the same z-position as MTs. Therefore the bead velocity was the same as the velocity of the fluid experienced by MTs. We calculated the average velocity of the flow for each frame. If beads could not be seen for a few frames (∼1–10), we interpolated the velocity between frames.

Measurement of single MTs/MT bundle rigidity. See Supplementary Methods and Supplemental Figure S6 for a detailed description.

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