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Role of nucleation in cortical microtubule array organization: variations on a theme

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Abstract

The interphase cortical microtubules (CMTs) of plant cells form strikingly ordered arrays in the absence of a dedicated microtubule-organizing center. Considerable effort has focused on activities such as bundling and severing that occur after CMT nucleation and are thought to be important for generating and maintaining ordered arrays. In this review, we focus on how nucleation impacts CMT array organization. The bulk of CMTs are initiated from γ-tubulin-containing nucleation complexes localized to the lateral walls of preexisting CMTs. These CMTs grow either at an acute angle or parallel to the preexisting CMT. Although the impact of microtubule-dependent nucleation is not fully understood, recent genetic, live-cell imaging and computer simulation studies have demonstrated that the location, timing and geometry of CMT nucleation have a large impact on the organization and orientation of the CMT array. These nucleation properties are defined by the composition, position and dynamics of γ-tubulin-containing nucleation complexes, which represent control points for the cell to regulate CMT array organization.

Introduction

Microtubules are a major component of the plant cytoskeleton, essential for many cellular processes including morphogenesis, cell division and intracellular trafficking. Microtubules are able to perform these different functions by organizing into distinctive arrays that act as a scaffold to guide molecular activities in space and time. During interphase, microtubules are found beneath the plasma membrane of plant cells. These so-called cortical microtubules— first imaged by Ledbetter and Porter in their seminal 1963 paper— serve to define cell shape by guiding the directional deposition of cell wall material (Szymanski and Cosgrove, 2009; Lloyd, 2011). To perform this morphogenetic function, cortical microtubules (CMTs) need be organized into specific patterns. These patterns can vary among cell types as well as in the same cell depending on developmental and environmental cues (Figure 1). For example, in rapidly elongating hypocotyl and root cells CMTs are organized such that they are coaligned with respect to each other and the array as a whole is typically oriented transverse to the cell elongation axis. When cell elongation slows down or stops, CMTs maintain their coaligned state but the array changes orientation to be along the cell elongation axis. Hindering or altering CMT organization, using drugs or through mutations, leads to abnormal plant growth and development, emphasizing the critical role of CMT organization to the life of a plant (Buschmann and Lloyd, 2008; Wasteneys and Ambrose, 2009).

In plants, CMTs form orderly patterns in the absence of focused nucleation centers like the centrosome and spindle pole body of animal and fungal cells. Instead, CMTs are initiated from multiple, dispersed sites at the cell cortex. How such a

decentralized system of microtubule generation contributes to CMT array organization is the focus of this review. For a review of the role of microtubule nucleation in mitotic plant microtubule arrays, see Masoud et al. (pp. aaa-bbb) in this issue.

The hardware for microtubule nucleation

Microtubules are highly dynamic polymers of αβ-tubulin continuously switching between periods of growth and shortening (Mitchison and Kirschner, 1984). *In vitro*, microtubules can self-assemble from purified αβ-tubulin subunits under appropriate conditions. However, in cells microtubule assembly is tightly regulated and requires nucleation factors to initiate new microtubules.

One of the key components of the microtubule nucleation machinery is a member of the tubulin protein family called γ-tubulin. The *Arabidopsis* genome contains two γ-tubulin genes (called *TubG1* and *TubG2*), which encode for proteins with 98% amino acid sequence identity (Liu *et al.*, 1994). Both genes are constitutively expressed throughout the plant and function redundantly (Binarova *et al.*, 2006; Pastuglia *et al.*, 2006). Genetic analyses show that absence of γ-tubulin results in severe developmental defects and embryo lethality (Binarova *et al.*, 2006; Pastuglia *et al.*, 2006). In addition, depletion of functional γ-tubulin severely impairs microtubule nucleation and is accompanied by disorganization and eventual loss of CMTs (Binarova *et al.*, 2006; Pastuglia *et al.*, 2006), indicating that γ-tubulin-based microtubule nucleation is essential for the creation and organization of CMT arrays.

Although γ-tubulin is essential for microtubule nucleation, by itself it is unable to initiate new microtubules. Rather, γ-tubulin associates with additional proteins to form

microtubule nucleating complexes. These γ-tubulin-interacting proteins are called γ-tubulin complex proteins or GCPs, where γ-tubulin itself is designated GCP1. The γ-tubulin in association with GCP2 and GCP3 forms a γ-tubulin small complex (γ-TuSC), which has relatively low nucleating activity (Oegema *et al.*, 1999). Multiple γ-TuSCs associate with additional proteins named GCP4, GCP5 and GCP6 to form a ring structure known as the γ-tubulin ring complex (γ-TuRC) (Zheng *et al.*, 1995). The γ-TuRC acts as a template for microtubule assembly and is a potent microtubule nucleator (Kollman *et al.*, 2011).

Plant genomes encode for the core GCP proteins that form the γ-TuRC (Schmit, 2002; Guo *et al.*, 2009). Therefore, it is likely that the basic mechanism for microtubule nucleation is conserved in plants. However, the assembly and structure of the plant γ-TuRC remain to be determined.

Connecting the dots: from γ-TuRCs to CMT nucleation

Live-cell analysis of the recovery pattern of CMTs after their initial drug-induced depolymerization showed that CMTs reassemble from multiple cortical sites (Wasteneys et al. 1993). Consistent with this observation, immunofluorescence microscopy revealed that γ-tubulin is not clustered at a specific site in plants cells but instead shows punctate localization along the length of CMTs (Liu *et al.*, 1993). Together, these observations indicated that CMT nucleation is likely to be dispersed throughout the cell cortex rather than being focused at a discrete location. Recent live-cell imaging studies have found that CMT nucleation is indeed dispersed throughout the cell cortex (Chan *et al.*, 2003; Shaw *et al.*, 2003). A careful analysis of CMT nucleation and its relationship to γ-tubulin

subsequently revealed that new CMTs predominantly emanate from the sides of existing CMTs in a branching pattern with γ-tubulin located at the branch points (Murata *et al.*, 2005). Branch-like CMT nucleation had been previously reported in *Nitella* cells (Wasteneys and Williamson, 1989a) and therefore branch-form CMT nucleation likely represents an ancient mechanism for plant CMT creation (Murata and Hasebe, 2007).

In animals, γ-TuRCs are assembled in the cytoplasm and subsequently recruited to the centrosome for microtubule nucleation. Biochemical fractionation and live-cell imaging studies show that plant γ-TuRCs exists as a cytoplasmic and membrane-bound pool (Drykova *et al.*, 2003; Seltzer *et al.*, 2007; Nakamura *et al.*, 2010). It is proposed that cytoplasmic γ-TuRCs gets recruited to the lateral walls of CMTs, upon which the bound γ-TuRCs are activated for microtubule nucleation (Murata *et al.*, 2005; Nakamura *et al.*, 2010). A γ-TuRC-associated protein called NEDD1 targets γ-TuRCs to the centrosome in animal cells (Luders *et al.*, 2006). Plants possess a NEDD1 homolog that co-localizes with γ-tubulin in mitotic arrays. NEDD1 shows a punctate distribution at the cell cortex of plant cells when transiently expressed and new CMTs originate from NEDD1-marked foci (Chan *et al.*, 2009; Zeng *et al.*, 2009). Together, these data indicate that NEDD1 may function to recruit γ-TuRCs to nucleation sites in plant cells.

In animals, augmin is reported to recruit γ-TuRCs to the lateral walls of pre-existing microtubules (Goshima et al. 2008). In plants, augmin has been shown to be important for the recruitment of γ-TuRCs to microtubules that make up the spindle and phragmoplast (Ho et al. 2011; Nakaoka et al. 2012). It is possible that augmin also recruits γ-TuRCs to CMTs during interphase, but this remains to be demonstrated.

Recently, another factor called GIP1 (GCP3-interacting protein 1) has emerged as a candidate for recruiting and/or activating γ-TuRCs bound to CMT side walls. The plant GIP1 proteins are homologous to vertebrate MOZART1, which is involved in recruiting γ-TuRCs to centrosomes during interphase and mitosis (Hutchins *et al.*, 2010). The *Arabidopsis* genome contains two GIP1-encoding genes called *GIP1a* and *GIP1b* (or *GIP2*) that function redundantly (Janski *et al.*, 2012; Nakamura *et al.*, 2012). GFP-tagged GIP1a localizes nearly exclusively along CMTs and these GIP1a-marked foci are associated with about 3-fold higher nucleating activity compared to GCP2/GCP3-marked foci along CMTs (Nakamura *et al.*, 2012). Pull-down of GFP-tagged GIP1a and GIP1b proteins recovers the core γ-TuRC proteins (i.e., γ-tubulin and GCP2-GCP6). In addition, both GIP1a and GIP1b are found to directly interact with the N-terminus of GCP3 (Janski *et al.*, 2012; Nakamura *et al.*, 2012). Together, these data are consistent with a role for GIP1a/b in recruiting γ-TuRCs to the CMT surface and/or activating CMT-bound γ-TuRCs through its interaction with GCP3. Structural analysis of γ-TuSCs suggests a conformational activation model in which a shift in the position of GCP3 in the γ-TuSC activates the nucleation complex (Kollman *et al.*, 2011). Since GIP1a/b directly binds to GCP3, this interaction could result in an activated conformation of the plant γ-TuRC on the CMT surface.

The high microtubule nucleation activity of GIP1a foci correlates with a lack of NEDD1 in the GIP1a-associated γ-tubulin complex (Nakamura *et al.*, 2012). In comparison, NEDD1 containing γ-tubulin complexes are associated with 3-fold lower nucleation activity (Nakamura *et al.*, 2010). These observations suggest that the

nucleation activity of γ-TuRCs can be modulated by different interacting proteins, possibly acting to tune nucleation in the cell. Where to insert Teixido-Traversa ref?

To branch or not to branch

Shown to be the dominant form of CMT nucleation, branch-form nucleation occurs when a new CMT is initiated at an acute angle along a pre-existing CMT (Figure 2A) (Wasteneys and Williamson, 1989a; Murata *et al.*, 2005; Chan *et al.*, 2009; Nakamura *et al.*, 2010). Measurements of the angle between the newly formed CMT and the pre-existing or "mother" CMT show a fairly wide distribution (20-60°) that centers around 40° (Murata *et al.*, 2005; Chan *et al.*, 2009; Nakamura *et al.*, 2010). Analogous to branch-form microtubule nucleation, the Arp2/3 complex nucleates actin filaments in a branch-form configuration. However, in this case the new actin filament is always formed at a distinctive angle of 70° with respect to the mother actin filament (Mullins *et al.*, 1998; Amann and Pollard, 2001). The Arp2/3 complex has a large binding interface with the mother actin filament that docks the Arp2/3 complex rigidly onto the mother filament (Rouiller *et al.*, 2008). This property of the Arp2/3 complex is thought to result in the observed invariant actin branching angle. While structural information is available for microtubules and γ-TuRCs, how γ-TuRCs dock onto the microtubule surface is unknown. The wider range of angles for CMT branch-form nucleation compared to its actin analog suggests that γ-TuRCs make less extensive and/or weak contact with the microtubule lattice, which might allow a tethered γ-TuRC to pivot to some extent (Figure 2B). Evidence from genetic and computer simulation studies show that the distribution of CMT branching angles has a large impact on CMT

array organization as discussed below.

Branch-form nucleation has been reported to have no significant preference for one side of the mother CMT. However, there is a clear bias for branching in the same direction as the plus-end of the mother CMT (Chan *et al.*, 2009). This finding suggests that docking of the γ-tubulin nucleation complex with the CMT lattice has a direction bias possibly arising from the inherent polarity of the microtubule lattice (Chan *et al.*, 2009).

In addition to branch-form nucleation, a second type of microtubule-dependent CMT nucleation has been observed in which a new CMT grows along the length of its mother CMT. This type of nucleation is called parallel-form nucleation (Figure 2A) and it occurs half as frequently as branch-form nucleation in wild-type plants (Chan *et al.*, 2009; Nakamura *et al.*, 2010). As in branch-form, parallel-form nucleation also has a preference for growth in the same direction as the preexisting CMT. Parallel-form nucleation effectively reinforces bundle formation within the CMT array, impacting the formation and reorganization of the CMT array as discussed later. The residence time of GCP2-labeled nucleation complex on CMTs is not significantly different between branch-form and parallel nucleation (Nakamura *et al.*, 2010). Therefore, the mechanism distinguishing these two modes of microtubule-dependent nucleation does not seem to depend on regulation of nucleation activity of an attached γ-TuRC. Rather, distinctive γ-TuRC-recruiting factors and/or regulatory events are likely to specify branch-form versus parallel-form nucleation (Figure 2B).

The least frequent type of CMT nucleation event, responsible for about 1-2% of all nucleation events, is referred to as free or *de novo* nucleation (Figure 2A). In this

case, a new CMT is initiated from a cortical area devoid of existing CMTs (Shaw *et al.*, 2003; Chan *et al.*, 2009; Nakamura *et al.*, 2010). It is unknown whether a distinctive mechanism for activating the γ-tubulin nucleation complex is required for free CMT nucleation. Given the rarity of free nucleation, perhaps free nucleation represents the chance activation of cortical γ-TuRCs rather than a specific activating factor.

The GFP-labeled markers that have been used to characterize CMT nucleation in living cells are listed in Table 1.

Relationship between nucleation and severing of CMTs

Plant CMTs are not stably attached to their nucleation sites but are released from these sites within 60s following nucleation by the microtubule severing protein katanin (Nakamura *et al.*, 2010). Therefore, γ-TuRCs do not act as microtubule minus-end caps in plants. Characterization of katanin mutants and computer simulation studies have both shown that inability to release CMTs from nucleation sites leads to poorly organized CMT arrays (Burk *et al.*, 2001; Allard *et al.*, 2010; Eren *et al.*, 2010). How katanin is specifically recruited to nucleation sites is an important open question. Since all CMTs appear to be released from their nucleation sites, the ability to sever CMTs does not seem to be sensitive to whether a CMT was generated via the branch-form, parallel-form or free nucleation mode.

One possible mechanism for targeting katanin to nucleation sites is a physical interaction, either direct or indirect, between katanin and γ-TuRCs. An interaction between katanin and γ-TuRCs would also be consistent with the observation that a significant fraction of new CMTs initiate from CMT crossover sites (Chan *et al.*, 2009),

known to be preferentially targeted for severing (Wightman and Turner, 2007).

How nucleation impacts CMT organization: insights from genetic analyses

As expected, many of the *Arabidopsis* mutants that affect CMT nucleation encode for components of the γ-TuRC (Table 2). These mutations impact different parameters such as nucleation efficiency, mode of nucleation (i.e., branch-form versus parallel-form) and distribution of initiation angle during branch-form nucleation. Notably, these perturbations alter CMT array organization in different ways, thus illustrating that CMT nucleation properties regulate CMT array organization in multiple ways.

As branch-form nucleation is dominant in the CMT array, it is expected to have a large contribution to array organization. Indeed, several mutant studies have illustrated that perturbation of branch-form nucleation impacts array organization. In the partial loss-of-function *gcp2/spr3* mutant, the distribution of branch-form angles is wider than observed in wild-type CMT arrays, leading to an increase of about 10° in the mean branching angle of the *spr3* mutant (Nakamura and Hashimoto, 2009). The CMTs in the *spr3* mutant form a left-handed oblique array instead of transverse arrays, suggesting that branch-form nucleation angle plays a role, either directly or indirectly, in defining CMT array orientation. Computer simulation studies have also suggested that significantly increasing the branching angle enhances the probability of forming oblique arrays (Eren *et al.*, 2010).

CMT nucleation frequency is unchanged in the *spr3* mutant (Nakamura and Hashimoto, 2009), indicating that this mutation does not reduce the nucleation competency of γ-TuRCs. The *spr3* mutation changes an invariant Gly to Arg in the

conserved GRIP1 motif of GCP2 (Nakamura and Hashimoto, 2009). A structural model of the γ-TuSC positions the GRIP1 motif of GCP2 towards the base of the γ-TuSC and facing the outside of the γ-TuRC structure (Kollman *et al.*, 2011). In this configuration, the GRIP1 domain of GCP2 would be available to interact with other proteins such as ones that dock the γ-TuRC to the CMT surface. Disruption of such an interaction might explain the more divergent branching angles in the *spr3* mutant.

In contrast to the *spr3* mutant, artificial microRNA-induced down regulation of *GCP4* expression leads to a decrease in the angle of branch-form nucleation, with the mean branch-form angle becoming about 25° compared to 40° in wild-type plants (Kong *et al.*, 2010). The CMTs in these plants are highly coaligned, perhaps because new CMTs that initiate at low branching angles have a greater chance of bundling with existing CMTs (Kong *et al.*, 2010). Repression of *GCP4* expression greatly reduces the amount of γ-tubulin localized at spindle poles and the phragmoplast, which are inferred to be nucleation sites during cell division. Instead, γ-tubulin localization is more diffuse in these cells, consistent with more γ-tubulin partitioning into the soluble cytosolic fraction (Kong *et al.*, 2010). These data suggest that GCP4 is important for proper γ-TuRC assembly and/or recruitment to nucleation sites. Whether *GCP4* repression displaces γ-tubulin from cortical sites during interphase was not reported. If *GCP4* repression results in less γ-tubulin along CMTs, then total nucleation frequency would be expected to be reduced in these plants.

Another class of nucleation mutants is exemplified by the *ton1* and *ton2*/*fass* mutations. TON1 and TON2/FASS are not considered to be integral components of γ-TuRCs, but likely represent regulatory factors. In the *ton2/fass* mutant, neither the

nucleation frequency nor the distribution of branching angles is significantly altered compared to wild-type plants (Kirik *et al.*, 2012). Instead, in the *ton2*/*fass* mutant, the proportion of parallel-form nucleation is greatly increased at the expense of branch-form nucleation (Kirik *et al.*, 2012). Therefore, TON2 activity is needed to promote branch-form nucleation in the CMT array. The CMT array in the *ton2* mutant is less dense and is unable to achieve light-induced array reorientation (Kirik *et al.*, 2012). These phenotypes indicate that wild-type levels of branch-form nucleation are important for both normal CMT array density and for remodeling of the CMT array.

TON2 encodes a B'' regulatory subunit of class 2A protein phosphatase (Camilleri *et al.*, 2002). Whether the effect of TON2 is through a direct interaction with the γ-TuRC or via modulation of the phosphorylation status of a γ-TuRC component remains unknown (Kirik *et al.*, 2012). Overexpression of TON2 that is engineered to be targeted to the plasma-membrane results in striking morphological defects in the trichome and leaf pavement cells (Kirik *et al.*, 2012). The CMT arrays are more coaligned in these plants, contrary to the expectation that increased TON2 activity would lead to more diverse CMT orientations because of increased branch-form nucleation. To address this issue, the branch-form nucleation frequency in plants overexpressing the plasma membrane-localized TON2 needs to be determined.

The *ton1* mutant of Arabidopsis shows severe morphological abnormalities that are associated with aberrant CMT organization and loss of the preprophase band (Azimzadeh et al. 2008). The *TON1* locus contains two nearly identical genes, *TON1a* and *TON1b*, which encode for small acidic proteins with similarity to the human centrosomal protein FOP (Azimzadeh et al. 2008). Recently, TON1 was found to

interact with a protein called TRM1, which shares similarity with the human centrosomal protein CAP350 (Drevensek *et al.*, 2012). CAP350 is responsible for recruiting FOP to the centrosome in humans. In plant cells, TRM1 interacts with both CMTs and TON1 and is proposed to recruit TON1 to CMTs (Drevensek et al. 2012). TON1 localizes along the length of CMTs in a punctate manner, reminiscent of the localization pattern of γ-tubulin (Azimzadeh et al. 2008). However, whether TON1 regulates CMT nucleation remains an open question.

How nucleation impacts CMT organization: insights from computer simulations

Several computer simulation models have probed the role of CMT nucleation in array organization (Allard *et al.*, 2010; Eren *et al.*, 2010; Deinum *et al.*, 2011). The simulations of Allard et al. 2010 and Eren et al. 2010 found that branch-form nucleation contributes to the density and polarity of the CMT array. In addition, as mentioned above, Eren et al. 2010 reported that the angle of branch-form nucleation significantly impacts the probability for forming skewed CMT arrays.

More recently, Deinum et al. 2011 conducted a thorough analysis of how different types of CMT nucleation impact CMT organization. They found that compared to simulations where free nucleation is the only mode of CMT initiation, using experimentally observed proportions of branch-form and parallel-form nucleation leads to better microtubule alignment, which occurs more quickly and over a wider range of CMT polymerization dynamics (Deinum *et al.*, 2011). Thus, microtubule-based nucleation appears to promote CMT alignment in multiple ways. In these computer simulation studies, the degree of microtubule alignment correlates with the proportion of

parallel-form nucleation (Deinum *et al.*, 2011). Therefore, while branch-form CMT nucleation is dominant in the CMT array, it is parallel-form nucleation that mainly contributes to CMT alignment. This conclusion is consistent with a conceptual model, which envisioned parallel-form nucleation to maintain array organization (Wasteneys and Ambrose, 2009). An increase in parallel-form nucleation, at the expense of branch-form nucleation, is predicted to reinforce the existing array orientation and resist array reorientation. This is because under these conditions most new CMTs would grow along existing CMTs rather than in new orientations. Consistent with this prediction, an increase in the proportion of parallel-form nucleation in the *ton2* mutant correlates with an inability to reorient the CMT array in response to blue light (Kirik *et al.*, 2012).

Branch-form nucleation is proposed to promote formation of a uniformly dense CMT array by creating new CMTs at divergent angles (Wasteneys and Ambrose, 2009; Deinum *et al.*, 2011). Analysis of CMT array recovery in *Nitella* cells after drug-induced depolymerization reveals that initially CMTs assemble in a transverse orientation. Soon thereafter, branch-form assembly dominates and CMTs become widely dispersed and show little alignment (Wasteneys and Williamson, 1989b). Subsequent organization of CMTs in these cells is associated with greatly reduced CMT branching (Wasteneys and Williamson, 1989b). These findings are consistent with the computer simulations of Deinum et al. 2011 in which branch-form nucleation leads to dispersal of CMTs, while parallel-form nucleation contributes to alignment of CMTs.

Are there microtubule-organizing centers in plants?

Although organization of the plant CMT array occurs in the absence of a

centrosome, the γ-tubulin containing nucleation complex has been observed to accumulate at specific locations from which new microtubules are initiated in a relatively focused manner. These clusters of γ-tubulin containing nucleation complex may represent non-canonical microtubule-organizing centers. Electron microscopic analysis of dividing cells in *Azolla* root meristems consistently showed CMTs growing out from the edges of newly formed cell walls, which was interpreted as microtubule nucleation from these cell edges (Gunning *et al*., 1978). Recent studies using diving *Arabidopsis* root cells show that GCP2-GFP-marked nucleation complexes accumulate preferentially along newly formed cell edges immediately following cell division and that this accumulation is lost over time (Ambrose and Wasteneys, 2011a). Microtubules have also been observed to emanate from the nuclear surface of *Haemanthus* and tobacco BY-2 cells (De May et al. 1982; Bajer and Mole-Bajer 1986). Consistent with these observations γ-tubulin containing nucleation complexes are found to accumulate on the nuclear surface in tobacco BY-2 cells (Yoneda and Hasezawa 2003; Erhardt *et al.*, 2002; Seltzer *et al.*, 2007). As CMTs are disassembled at the onset of mitosis, it is proposed that nucleation from cell edges and the nuclear surface serves to repopulate CMTs after cytokinesis (Yoneda and Hasezawa, 2003; Ambrose and Wasteneys, 2011b). The mechanisms for the formation and dissolution of these focused nucleation sites in plant cells remain unknown. In addition, whether γ-tubulin localization at cell edges and on the nuclear surface is important for the genesis of the CMT array is still an open question.

Conclusions and future directions

Based on the evidence reviewed here, it is clear that nucleation activity is not merely a source of new CMTs (to replace CMTs that are lost due to depolymerization), but that it serves an important regulatory function to fine-tune the degree of CMT alignment and overall array orientation. In particular, nucleation from γ-tubulin containing complexes allows control of the location, timing and geometry of CMT nucleation—factors that can be varied to generate particular CMT array patterns and to remodel them. Specifically, variation in the proteins involved in nucleation may contribute to the rate, efficiency, and geometry of nucleation. In contrast, variation in the localization of the nucleating complex may influence the overall array orientation. These variations on the theme of nucleation are likely to be important for generating the diversity of CMT patterns observed in plants.

The finding that microtubule-based nucleation is the dominant form of CMT assembly raises the possibility that nucleation and array organization might feedback on each other (Deinum *et al.*, 2011). For example, if CMT alignment increases the frequency of parallel-form nucleation or decreases the branching angle along the aligned CMTs, this positive feedback would work to reinforce and maintain the existing CMT pattern. In contrast, an increase in branch-form nucleation would promote loss of CMT organization, perhaps as a precursor to a different organized state. Based on mutant analyses, the relative proportion of branch-form and parallel-form nucleation and the distribution of branch-form angles have emerged as important control points that affect the balance between CMT alignment and dispersal.

Molecular genetic analyses, live-cell imaging, biochemistry and computer simulations together have provided critical insights into the composition, location,

dynamics and function of the plant microtubule nucleation complexes. A combination of these approaches promises to be a powerful tool to address many remaining questions such as:

1. What are the molecular mechanisms that specify the location, timing and geometry of CMT nucleation? Mutant analysis has identified TON2, GCP2/SPR3, GCP4 and GIP1a/b as factors that promote branch-form nucleation or regulate the branching angle. However, factors involved in inducing parallel-form nucleation and for localizing γ-tubulin containing nucleation complexes to cell edges and the nuclear surface remain to be identified.

2. What is the contribution of nucleation to CMT array organization in different cell types? The best CMT nucleation data are available for hypocotyl cells, in which CMTs are organized into relatively simple linear arrays. However, CMT arrays adopt very different patterns in other cell types such as leaf pavement cells, xylem vessels and tip growing cells. Whether and how nucleation affects the formation of CMT arrays in these cell types remain open questions. In this regard, it is known that in guard cells γ-tubulin accumulates at the cortical surface facing the stomatal pore and that CMTs emanate mainly from this surface forming a radial array (Galatis *et al.*, 1983; McDonald *et al.*, 1993). Thus, how nucleation is regulated in space and time can vary significantly among different cell types.

3. How does nucleation relate to other CMT behaviors such as bundling, severing, and membrane attachment? While we have focused on nucleation in this review, this process works in concert with other CMT activities that are known to be important for the organization and orientation of the CMT array. Nucleation could indirectly impact

other CMT activities by affecting the available tubulin subunit pool. The tubulin subunit pool would predictably alter the polymerization dynamics of CMTs, in turn impacting the frequency of bundling, severing and the attachment of CMTs to the plasma membrane. However, nucleation could also more directly affect other CMT activities. As discussed earlier, there is circumstantial evidence for a mechanistic link between nucleation and severing. Similar links could exist between nucleation and bundling as well as between nucleation and plasma membrane attachment. For example, bundling may promote parallel-form nucleation and stronger attachment of CMTs to the plasma membrane may increase recruitment of nucleating complexes to these CMTs. With the availability of nucleation site markers for live-cell imaging, it should be possible to determine if nucleation is correlated to these CMT behaviors as a first step towards defining interactions among these disparate CMT activities.

Conflict of interest

The authors have no conflict of interest.

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Table 1: Markers used for live-cell imaging of CMT nucleation

Marker name	Structure labeled	Branch-form angle (in wild-type background)	Reference
GFP-TUA6	Microtubule polymer	41.6 ± 8.2° (BY-2 cells) 36.0 ± 10.7° (cell-free)	Murata *et al.*, 2005
EB1a-GFP	Growing plus-ends	Mean of 55° (right-side) Mean of 44° (left-side)	Chan *et al.*, 2009
GFP-TUB6	Microtubule polymer	38.6 ± 4.6° (hypocotyl) 41.4 ± 8.1° (cotyledon) 41.0 ± 10.2° (leaf)	Nakamura and Hashimoto, 2009; Kong *et al.*, 2010
GCP2-GFP/ GCP3-GFP	Nucleation site	39.6 ± 5.9° (hypocotyl)	Nakamura *et al.*, 2010; Kirik *et al.*, 2012

Table 2: *Arabidopsis* mutants with altered CMT nucleation properties

Mutant name	Nucleation frequency (μm^{-2} min^{-1})	Nucleation type ratio	Branch-angle	Reference
gcp2/spr3	3.25 x 10^{-3}	Not determined	49 ± 11° (hypocotyl) 49 ± 13° (cotyledon)	Nakamura and Hashimoto, 2009
amiRNA-*GCP4*	Not determined	Not determined	27 ± 11° (leaf)	Kong *et al.*, 2010
ton2/fass	2.02 x 10^{-3}	12% branch-form 72% parallel-form 16% free	Mean of 44° (leaf)	Kirik *et al.*, 2012
gip1a/*gip1b*	Not determined	Not determined	Not determined	Nakamura *et al.*, 2012

Figure Legends

Figure 1: Examples of CMT array patterns in an *Arabidopsis* seedling. At the zone of rapid cell elongation near the apex of the hypocotyl, CMT arrays in epidermal cells show net transverse orientation. As the cells stop elongating and mature, towards the base of the hypocotyl, CMT arrays in epidermal cells show net longitudinal orientation. In cotyledons and leaves, CMT arrays show a complex, net-like pattern in pavement cells and a radial pattern in guard cells.

Figure 2: Types of CMT nucleation. (A) In branch-form nucleation, a new CMT initiates from the surface of a mother CMT at an acute angle. In parallel-form nucleation, a new CMT initiates from the surface of a mother CMT in a coaligned manner. In free nucleation, a new CMT initiates at the cell cortex independently of preexisting CMTs. Black circles represent γ-tubulin containing nucleation complex. In the case of free nucleation, it is not known whether the nucleation complex is bound to the plasma membrane. (B) Hypothetical mechanisms for branch-form and parallel-form nucleation. In these diagrams, the γ-tubulin containing nucleation complex is depicted as a purple cone, the mother CMT is colored blue and the newly initiated CMT is colored green. During branch-form nucleation, it is envisioned that the γ-tubulin containing nucleation complex is bound to the surface of the mother CMT relatively weakly. A weakly tethered nucleation complex might pivot within an angular range that centers around 40° in wild-type cells. During parallel-form nucleation, the γ-tubulin containing nucleation complex is envisioned to be more tightly bound to the mother CMT surface possibly due to

distinct recruiting factors that extensively bind to the mother CMT or by additional proteins that prevent pivoting of the nucleation complex.