Microtubule nucleation and organization without centrosomes

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Abstract

Centrosomes play various critical roles in animal cells such as microtubule nucleation and stabilization, mitotic spindle morphogenesis, and spindle orientation. Land plants have lost centrosomes and yet must execute many of these functions. Recent studies have revealed the crucial roles played by morphologically distinct cytoplasmic microtubule-organizing centers (MTOCs) in initiating spindle bipolarity and maintaining spindle orientation robustness. These MTOCs resemble centrosomes in many aspects, implying an evolutionary divergence of MT-organizing structures in plants. However, their functions rely on conserved nucleation and amplification mechanisms, indicating a similarity in MT network establishment between animals and plants. Moreover, recent characterization of a plant-specific MT minus-end tracking protein suggests that plants have developed functionally equivalent modules to stabilize and organize MTs at minus ends. These findings support the theory that plants overcome centrosome loss by utilizing modified but substantially conserved mechanisms to organize MT networks.

Introduction

Microtubules (MTs) are fundamental cytoskeletal elements that play critical roles in cell morphogenesis, division, and physiology. In live cells, MTs exhibit high dynamicity, such as growth, catastrophe, severing, bundling, and transport; these processes are precisely controlled to fulfill diverse functions [1] (Figure 1). Animal somatic cells employ centrosomes as the major microtubule-organizing centers (MTOCs) to arrange MTs during both interphase and mitosis [2]. However, land plant cells assemble well-organized

interphase MTs and mitotic spindles in the absence of centrosomes. How MTs are organized in the absence of centrosomes is a longstanding question.

Recent studies have revealed that many aspects of centrosome functions can be achieved in plants. Furthermore, the organization of MTs in plants and animals is similar at molecular and subcellular levels. In this review, we focus on three processes in which centrosomes play a major role in animals: (1) Plants assemble cytoplasmic MTOCs to initiate spindle bipolarity prior to nuclear envelope breakdown. These MTOCs are non-essential for spindle assembly, but are important for spindle orientation. (2) MT-dependent MT nucleation has been established as the key mechanism of MT generation, which enables generation of branched or parallel MTs on the lattice of pre-existing MTs. This mode of nucleation is suitable for rapid organization of the MT network in the absence of predominant MTOCs. However, different types of nucleation have apparently been observed in certain cell types. (3) MT dynamics at the minus ends are regulated by minus-end tracking proteins, suggesting that MT-associated proteins (MAPs) may autonomously regulate MT dynamics and contribute to MT network organization.

Spindle orientation without centrosomes

One of the major roles of centrosomes is to initiate spindle bipolarity in early mitosis. In plants, the cortical structure preprophase band (PPB), a ring-shaped MT array encircling the nucleus, has been proposed as a centrosome analogue based on its function and the homology between centrosomal proteins and proteins required for PPB formation [3]. This possibility is supported by observations that nuclear positioning and spindle bipolarity establishment in prophase are regulated by “bridge MTs”, which connect the PPB and nuclear envelope [4], and that multipolar spindle formation is correlated with double-PPB inducement [5]. However, mutant analyses have thrown into question the essentiality of PPBs in division, as both the spindle and phragmoplast appear normal in the absence of PPBs [6-9]. Because mutant plants lacking PPBs exhibit severe developmental defects accompanied with interphase MT disorganization [6-10], the essentiality and precise roles of PPB in mitosis remain unclear. Recently, Schaefer et al. isolated an *Arabidopsis trm* (TON1 Recruiting Motif) mutant that specifically abolishes PPB formation without affecting interphase MT organization [11]. Surprisingly, *trm* mutant plant growth was normal with only some loss of growth capacity and developmental robustness. Spindle bipolarity establishment was not affected either; however, the orientation of the spindle became variable.

In some plant cell types, such as endosperm cells in flowering plants and caulonemal cells in the moss *Physcomitrella patens*, the PPB is never formed [12,13]. In assembling spindles in plants, cytoplasmic MTs appear to be a

common key player. Cytoplasmic MTs can be polymerized and anchored to less-defined subcellular structures in the prophase stage and contribute to spindle assembly and orientation in many cell types. In seed plants, enriched cytoplasmic MTs are observed in pools surrounding the nuclear envelope during prophase [12,14-16], which later transform into bipolar structures termed polar caps or pro-spindles [15,17,18] (Figure 2b). Polar cap formation on opposite sides of the nuclear envelope is ensured by the PPB in PPB-forming cells [4,11,18]. Extensive data indicate that polar caps may, at least in part, play a similar role to centrosomes in organizing spindle bipolarity. First, MTs converge to the cap polar region [16]. Second, γ-tubulin, the major MT nucleator, is enriched at polar caps [17]. Third, MTs exhibit plus-end-out orientation around the poles [18]. Furthermore, analogous to animal centrosomes, polar caps are shown non-essential for spindle assembly, but required for proper orientation of the spindle and division plane [11,18-20] (Figure 2a and b).

The organization of cytoplasmic MTs into polar cap-like structures has also been observed in bryophytes. These structures participate in prophase spindle formation in a similar way. In moss caulonemal cells, nucleus-associated MT enrichment is observed during prophase [13,21]. MTs are unevenly distributed around the nucleus, ensuring that the assembled bipolar spindles are oriented parallel to the cell's long axis [21]. Following nuclear envelope breakdown, these MTs are integrated into the forming spindle [21]. Recently, another cytoplasmic MTOC structure has been characterized in the moss gametophore. Using time-lapse imaging, Kosetsu et al. found that the PPB is absent during gametophore initial cell division; instead, an MT cloud, termed the "gametosome", is observed in the cytoplasm at the apical side [20] (Figure 2c). This MT cloud is loosely focused with fluorescence signals extending outwards. During late prophase, the cloud migrates towards the nucleus and merges into the spindle MTs following nuclear envelope breakdown. Gametosome disruption experiments have shown that it is required for spindle orientation, but is non-essential for spindle assembly. Its formation requires γ-tubulin, but not actin or the TTP (TON1-TRM-PP2A) complex, which organizes interphase MTs and assembles PPB in seed plants. Based on its function, the gametosome is similar to polar caps in flowering plants.

Polar organizers (POs), a pair of centrosome-like structures located at opposite sides of the elongated nucleus during prophase, initiate mitotic spindle formation in liverworts [22,23]. Enriched γ-tubulin localization and the presence of astral MTs support the suggestion that POs are functional MTOCs [22-24]. However, POs do not contain centrioles and are not maintained during metaphase [22]. In the liverwort *Marchantia polymorpha*, both POs and PPBs arise in cells preparing for mitosis [23]. Interestingly, POs emerge prior to PPB formation and extra POs are correlated with the formation of disorganized

PPBs [23,25,26], suggesting that unlike polar caps, POs may act upstream or independent of PPBs [23]. In the hornwort, the cytoplasmic MTOC structure, the axial MT system (AMS), also marks spindle bipolarity during prophase [24,25,27]. AMS is associated with a single plastid and contains enriched γ-tubulin. However, it appears later than PPB formation, similar to polar caps or gametosomes in PPB-containing cells [25,27].

Despite structural differences, the polar cap, gametosome, PO, and AMS all resemble centrosomes in terms of initiating spindle bipolarity at early mitotic stages (Figure 2). They are similar in many aspects: 1. they emerge or develop in prophase; 2. they supply MTs through nucleation factor-mediated polymerization; 3. they are important for spindle orientation; and 4. they are non-essential for spindle assembly (note: this has not been experimentally shown for POs and AMS). These findings suggest that diverse cytoplasmic MTOCs have evolved to regulate spindle orientation in plants, which may compensate for centrosome loss. The non-essentiality of centrosomes and these cytoplasmic MTOCs is likely due to the existence of the conserved chromatin-triggered self-assembly mechanism that contributes to robust spindle assembly following nuclear envelope breakdown [28,29]. However, preset bipolarity in prophase can facilitate spindle assembly and orientation, which improves mitotic fidelity [19].

Microtubule nucleation without centrosomes

MT nucleation in both plants and animals is dependent on the γ-tubulin ring complex (γ-TuRC) [30]. In the absence of conspicuous MTOCs, the MTs in plants are predominantly generated by an MT-dependent nucleation mechanism, in which γ-TuRC localizes to the lateral surface of pre-existing MTs (mother MTs) and nucleates polymerization of new MTs (daughter MTs) [31]. This mechanism has been best studied in the cortical MT networks of flowering plants where it is thought to be the major system for MT population amplification [32,33]. For example, in *Arabidopsis* hypocotyl cells, only 1.4% of nucleation events are independent of MT-association [34]. Nucleation probability significantly increases when γ-TuRC is attached to extant MTs [34]. Because the orientation of daughter MTs is biased at an angle of ~40° or 0° from the mother MTs [31,34,35], MT-dependent nucleation is well suited for generating organized parallel MTs in the cortex [33]. *De novo* MT nucleation is also believed to occur, albeit infrequently, on the plasma membrane, where γ-TuRC is likely recruited through an MT-independent mechanism [32,34]. However, it is unknown how this process occurs. A recent study has reported that occasionally, after nucleating a 1.2 μm-long MT, γ-TuRC is pushed away towards the minus-end direction [36]. It is thought that the plus end is transiently anchored to the plasma membrane and continues to polymerize, leading to the repositioning of minus ends [36]. Subsequently, γ-TuRC disassociates from the minus ends. The depolymerization of minus ends and

resumed plus-end growth result in treadmilling. Recently, the augmin complex, which consists of eight subunits, has been shown to initiate branching nucleation of interphase cortical MTs, which is in agreement with its role in recruiting γ-TuRC and may explain how γ-TuRC is targeted to MT lattices for nucleation [37,38]. Interestingly, the involvement of augmin and γ-TuRC in polarized MT nucleation has also been demonstrated in neurons, revealing a similarity in the underlying mechanism of non-centrosomal MT network organization between animals and plants [39].

During mitosis, γ-TuRC is essential for the assembly of three types of distinct MT arrays, including the polar cap/gametosome, mitotic spindle, and phragmoplast [40]. Perturbing augmin complex function causes abnormal spindles, reduction of MT mass, less-converged kinetochore fibers, and mitotic delay, accompanied with decreased enrichment and altered localization patterns of γ-TuRC in *Arabidopsis* and *P. patens* [21,41-43]. These findings indicate a similar role of augmin complex in γ-TuRC recruitment during mitosis in both animals and plants [44,45], and that MT-dependent nucleation is a general mechanism in plants utilized for the establishment of diverse organized MT networks [38]. However, γ-TuRC does not always work together with augmin. For example, although augmin plays a dominant role in phragmoplast MT generation, no defects in polar cap/gametosome MT formation have been observed in *P. patens* [20]. In contrast, γ-TuRC is essential for MT formation throughout mitosis, likely because of its capability to nucleate MT in an MT-independent manner [20,21].

Another novel mechanism of MT nucleation was recently identified in *P. patens* protonemal cells, which do not form cortical MT arrays. MT nucleation spontaneously occurs in the interphase cytoplasm at random locations; interestingly, in ~20% of cases, γ-tubulin is undetectable at the MT minus end [46]. MT-dependent branching has also been observed; however, it occurs independent of augmin and the branching angle is variable [46]. MT nucleation has been studied in limited numbers of cell types; thus, there might be other unidentified modes of MT nucleation in plants.

Microtubule minus-end stabilization without centrosomes

Another major role of centrosomes in MT organization is to anchor and stabilize MT minus ends during both interphase and mitosis. In flowering plants, the plus ends of cortical MTs exhibit dynamic instability, while the free minus ends undergo slow depolymerization [47], indicating that the minus ends are protected. In animals, several centrosome-independent minus-end targeting and stabilizing proteins, such as CAMSAP/Patronin/Nezha family members, have been identified [48]. However, CAMSAPs are not present in the genomes of plants [49].

Recently, the plant-specific MT-binding protein SPIRAL2 (SPR2) has been identified as an MT minus-end targeting protein [50-52]. In *Arabidopsis*, SPR2 is predominantly localized to the minus ends and crossovers of cortical MTs [50,52]. It decorates and tracks both minus and plus ends when treadmilling occurs after severing at crossovers. However, SPR2 only labels the minus ends *in vitro*, supporting its role as a minus-end targeting protein [52]. Indeed, SPR2 is required for minus-end stabilization; loss of its function causes enhanced minus-end depolymerization and reduced severing probability at crossovers, which results in delayed MT reorientation induced by light stimulus [50,52]. *P. patens* SPR2 specifically localizes to and stabilizes the minus ends in protonemal cells [51]. It is possible that, SPR2 is recruited to the plus ends by other plus-end tracking proteins and plays additional roles that facilitate MT network remodeling in flowering plants, but not in moss protonemal cells, which form non-cortical MT networks. In addition, SPR2 may function in regulating MT dynamics during mitosis because it also localizes to the PPB, metaphase spindle, and phragmoplast [51,53,54], although functional analysis has not been reported.

In mammalian cells, katanin and ASPM (the abnormal spindle-like microcephaly-associated protein) form a complex to regulate MT minus-end dynamics at spindle poles [55]. ASPM protects MT minus ends and promotes katanin-mediated severing. In turn, katanin potentiates the minus-end blocking activity of ASPM. In plants, severing of cortical MTs at crossovers is triggered by katanin and facilitated by SPR2 [50,52,56-59]. It is intriguing to speculate that ASPM, which has not been characterized in plants, also coordinates with katanin and SPR2 in regulating MT dynamics. Whether other minus-end targeting proteins, including Msd1/SSX2IP and microspherule [49,60,61], play a similar role in plants, remains to be determined.

Modeling MT self-organization

Other molecules that regulate MT network assembly include plus-end stabilizing proteins and kinesin motors. The cytoplasmic linker associated protein (CLASP) has been shown to promote the assembly of an intricate cortical MT network via stabilizing the plus ends of transfacial MTs in *Arabidopsis* root cells [62]. Distinct kinesin-14 family members, such as ATK, KCBP and KCH, are known to transport or bundle MTs [63-65]. The nucleation factor augmin complex has been shown to antagonize katanin-mediated severing at crossovers [66]. Together with other MAPs, these factors are believed to control MT dynamics and facilitate MT self-organization, therefore driving the formation or remodeling of MT patterns in the absence of MTOCs [67]. However, how this process occurs remains mysterious. In recent years, advanced approaches including quantitative analysis of MT dynamics and computational modeling have come into focus, which enable deep investigations of synergistic effects on MT dynamics and network modeling. A

recent study, for example, shows that katanin-mediated selective severing of MTs at crossovers, but not at random locations, promotes alignment of cortical MTs, which supports *in vivo* observations [57-59]. This process is aided by the minus-end stabilizing factor SPR2 as revealed by quantitative imaging *in vivo* and biochemical assays *in vitro* [50,52]. By simulating MT dynamics *in silico*, two studies indicate that the orientation of cortical MT arrays is highly influenced by the cell shape [68,69]. As many factors such as nucleation site, plus- and minus-end stability, MT movement, and MT-MT interactions are regulated by specific sets of proteins *in vivo*, a combination of quantitative imaging and modeling in given genetic backgrounds is now used to investigate their actions on MT dynamics [50,62,66].

Concluding remarks

Over the past several years, studies using multiple model plant species have improved our understanding of how MTs are nucleated, stabilized, and organized in the absence of centrosomes in land plants. Notably, non-centrosomal MTOCs, gametosomes, and polar caps have been identified and/or experimentally characterized as the functional analogues of centrosomes. At a molecular level, conserved proteins, such as augmin or γ-TuRC, act on MTs via a mechanism common in plants and animals, whereas minus-end stabilizers (CAMSAP and SPR2) have uniquely evolved in each kingdom. Moreover, experimental analyses and computational modeling have revealed the critical role of self-organization in shaping MT networks in plants. Further studies addressing the roles of conserved MT-interacting molecules in the framework of a quantitative self-organization model will greatly advance our mechanistic understanding of MT organization in various plant cell types.

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- • of special interest
- •• of outstanding interest

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Short summary of selected papers:

•• **Schaefer et al., 2017:** This study isolated a *trm* mutant that specifically abolishes the formation of PPB and polar caps without affecting interphase microtubules in *Arabidopsis* and demonstrated that PPB and polar caps are non-essential for spindle assembly and cell division, but are required for accurate spindle orientation.

•• **Kosetsu et al., 2017:** This study identified centrosome-like MTOCs, termed “gametosomes”, in moss *P. patens* gametophores and revealed that they are crucial for spindle orientation, but are non-essential for spindle assembly. They also showed that the polar cap of flowering plants has an analogous role to gametosomes.

• **Buschmann et al., 2016:** This study reported the detailed characterization of polar organizers in the liverwort *Marchantia polymorpha* using live cell imaging and showed that they are centrosome-like MTOCs.

•• **Fan et al., 2018; Leong et al., 2018; Nakamura et al., 2018:** These three papers characterized the plant-specific protein SPIRAL2 as the first MT minus-end-tracking protein in plants. SPIRAL2 binds and stabilizes MT minus ends both *in vivo* and *in vitro*. In *Arabidopsis*, SPIRAL2 also localizes to MT crossovers and promotes katanin-mediated severing.

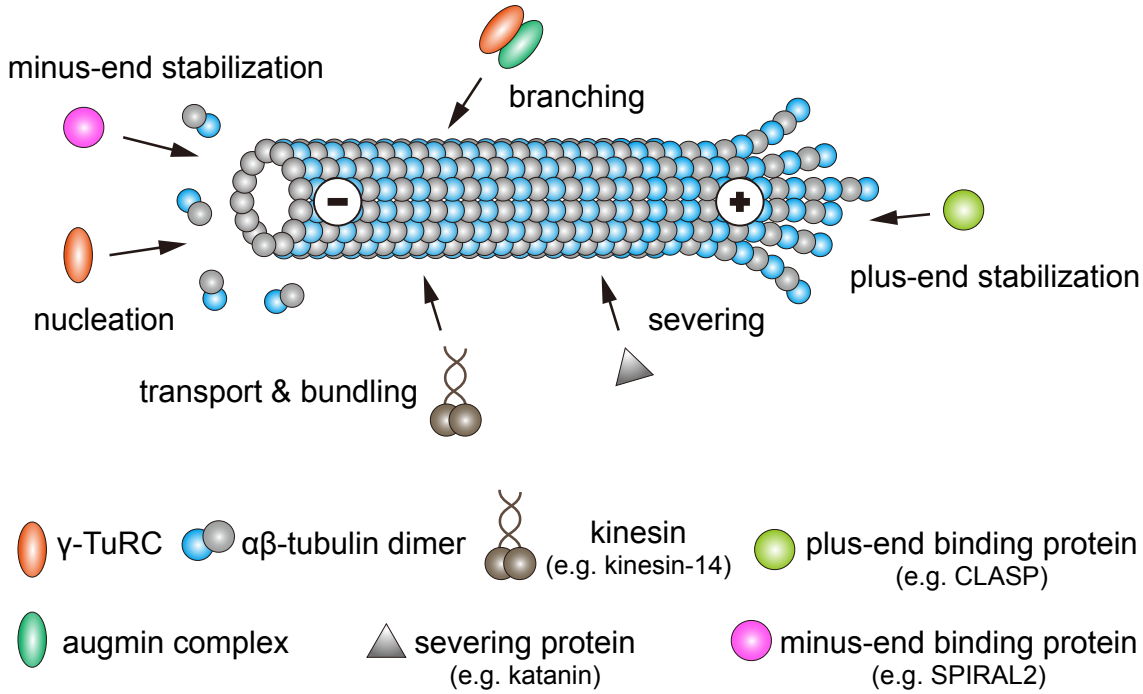
Figure legends

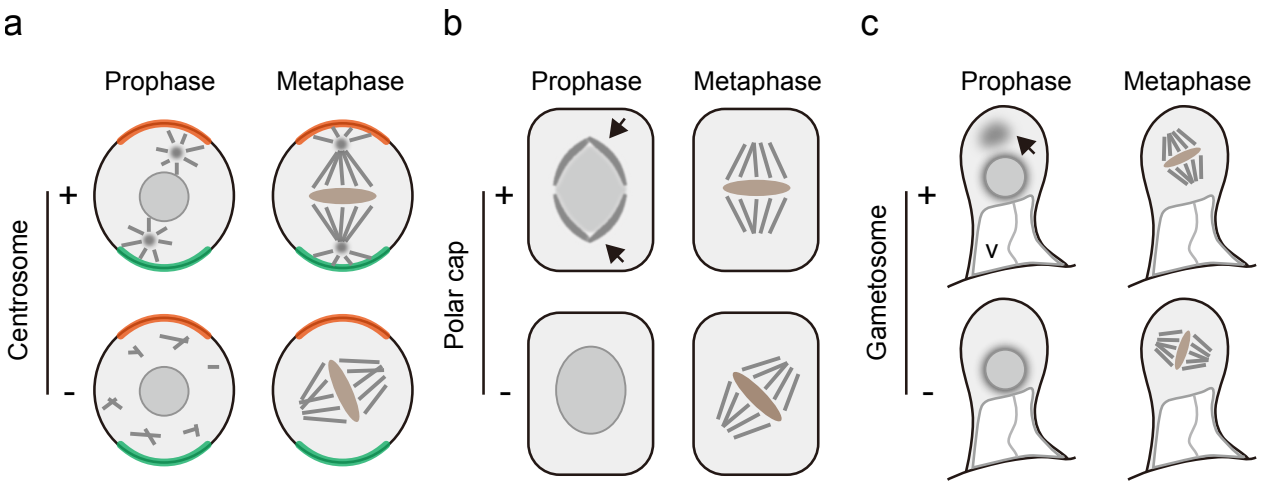
Figure 1: Regulation of microtubule dynamics

Microtubules (MTs) are filamentous polymers consisting of α- and β-tubulin dimers. They are mostly assembled through γ-tubulin ring complex (γ-TuRC)-triggered nucleation at the minus end (-) and subsequent addition of tubulin dimers at the plus end (+). MTs are intrinsically dynamic. In cells, MTs are assembled into distinct network structures at each phase of the cell cycle, which are essential for cell morphogenesis, division, and physiology. The arrangement of these networks is achieved by the regulation of MT dynamics, such as γ-TuRC-dependent nucleation, augmin- and γ-TuRC-mediated branching nucleation on the lateral surface, minus- and plus- end stabilization, severing, and transport and bundling by kinesin motors. These processes are highly conserved in animals and plants, but some factors involved evolved distinctly in animal and plant lineages. Representative regulatory molecules in plants are indicated in brackets.

Figure 2: Centrosome-like MTOCs are involved in spindle orientation in plants

a. Spindle orientation in the presence or absence of centrosomes in *Drosophila* neuroblasts [70,71]. Spindles are consistently oriented along the polarity axis in wild-type cells, but not in centrosome-depleted cells. Orange and green indicate asymmetric localization of membrane-associated polarity proteins, chromosomes are in light brown, and microtubules are in dark grey.
b. Spindle orientation in the presence or absence of polar caps in *Arabidopsis*. [11]. The polar caps, comprised of enriched microtubules (dark grey, indicated by arrows) surrounding the nucleus, exhibit bipolarity in late prophase. Absence of polar caps is associated with the deviation of spindle orientation.
c. Spindle orientation in the presence or absence of gametosomes in the moss *P. patens*. The gametosome is an MT cloud (dark grey, indicated by an arrow) that forms on the apical side of gametophore initial cells. In wild-type cells, the spindle is consistently orientated at an angle of ~30° along the apical-basal axis. This angle becomes variable when gametosome formation is blocked [20]. “v” indicates a large vacuole in the basal cytoplasm.


minus-end stabilization
branching
plus-end stabilization
nucleation
severing
transport & bundling
γ-TuRC
αβ-tubulin dimer
kinesin
(e.g. kinesin-14)
plus-end binding protein
(e.g. CLASP)
augmin complex
severing protein
(e.g. katanin)
minus-end binding protein
(e.g. SPIRAL2)


a
Prophase
Metaphase
Centrosome
+
-
b
Prophase
Metaphase
Polar cap
+
-
c
Prophase
Metaphase
Gametosome
+
-
v