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The rise and fall of the phragmoplast microtubule array

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The cytokinetic apparatus, the phragmoplast, contains a bipolar microtubule (MT) framework that has the MT plus ends concentrated at or near the division site. This anti-parallel MT array provides tracks for the transport of Golgi-derived vesicles toward the plus ends so that materials enclosed are subsequently deposited at the division site. Here we will discuss a proposed model of the centrifugal expansion of the phragmoplast that takes place concomitantly with the assembly of the cell plate, the ultimate product of vesicle fusion. The expansion is a result of continuous MT assembly at the phragmoplast periphery while the MTs toward the center of the phragmoplast are disassembled. These events are the result of MT-dependent MT polymerization, bundling of anti-parallel MTs coming from opposite sides of the division plane that occurs selectively at the phragmoplast periphery, positioning of the plus ends of cross-linked MTs at or near the division site by establishing a minimal MT-overlapping zone, and debundling of anti-parallel MTs that is triggered by phosphorylation of MT-associated proteins. The debundled MTs are disassembled at last by factors including the MT severing enzyme katanin.

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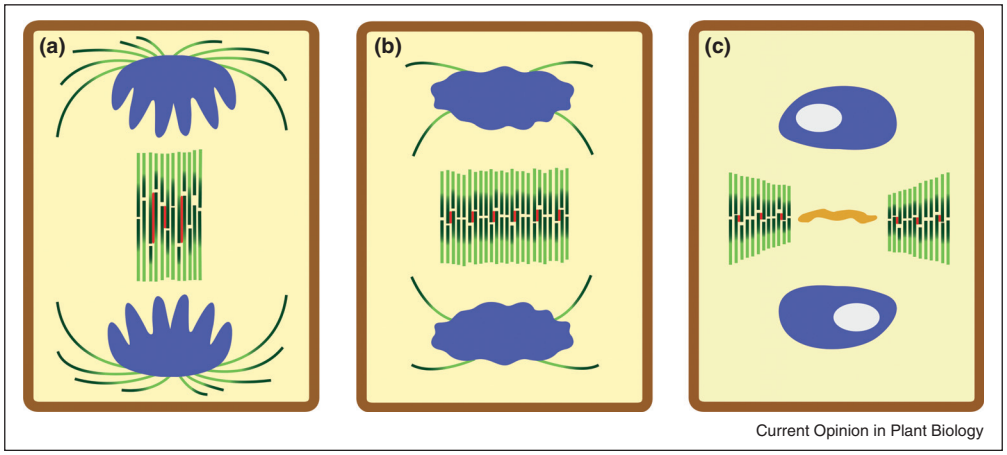
Introduction

The innovation of the phragmoplast marks a significant advancement in the evolution from green algae to land plants [1]. The phragmoplast contains a core of two mirrored sets of anti-parallel microtubules (MTs) whose plus ends are concentrated at or near the midzone of this cytokinetic apparatus (Figure 1) [2]. The ultimate mission of this bipolar MT array is to allow Golgi-derived vesicles to be transported unidirectionally toward MT plus ends so that the materials enclosed in these vesicles are deposited for the assembly of the cell plate. Phragmoplast MTs, like those MTs in spindles during mitosis, undergo continuous remodeling throughout cytokinesis [3,4]. Upon the completion of anaphase, MTs are polymerized and coalesced in the spindle midzone, and a bipolar array is established as the Kinesin-5 motor acts on anti-parallel MTs to slide them against each other (Figure 1a) [5,6••,7]. Concomitant with the addition of more MTs to the array, the MTs are shortened at their minus ends, which are facing the reforming daughter nuclei (Figure 1b). One of the most spectacular phenomena in cytokinesis is the centrifugal expansion of the phragmoplast MT array from the cell center to the edge. During the expansion, new MT filaments are added to the periphery of the phragmoplast while older ones in the inner part of the phragmoplast array are disassembled (Figure 1c). This is particularly challenging because those opposing activities occur simultaneously within a few microns of each other. The assembly of new MTs uses tubulin subunits released from the depolymerization of older MTs [3].

Before MT-associated proteins (MAPs) and MT-based motors were identified, microscopic observations had revealed many structural details of the phragmoplast in elegant systems like the endosperm of the African blood lily *Haemanthus* and tobacco BY-2 cells [8,9]. To date, those magnificent images still inspire us to dig into mechanisms underlying plant cytokinesis. In the past two decades or so, a mechanistic understanding of cytokinesis has been greatly advanced in the model system *Arabidopsis thaliana* by utilizing its powerful genetics together with live-cell imaging. Particularly, mutational analyses have led to the identification of proteins important for cytokinesis, many of which are conserved among eukaryotes [10,11]. Inactivation of a number of factors that regulate fundamental aspects of MT dynamics certainly leads to the eventual failure in cytokinesis [12]. However, there are factors that act specifically on phragmoplast MTs and regulate the rise and fall of the phragmoplast MT array [13].

While a number of insightful reviews have summarized the role of genes and proteins that regulate phragmoplast organization and cytokinesis [13–16], here we will focus on several critical MT reorganization events during phragmoplast expansion. We envisage that the phragmoplast MT array is assembled by anti-parallel MT modules of mini-phragmoplasts which contain a core of interdigitating MT bundles surrounded by non-interdigitating MTs [6••]. The centrifugal expansion of the array is brought about by the amplification of the mini-phragmoplast modules toward the periphery and the disassembly of old ones at the inner part of the phragmoplast, where the cell plate is formed. Figure 2 illustrates hypothesized sequential events that take place after interdigitating

Fig. 1



Phragmoplast MT arrays at representative stages. In the diagrams, MTs are shown in gradient green lines with the darkest representing MT plus ends and lightest for minus ends; interdigitating MTs are cross-linked by factors in red; nuclei in blue and nucleoli in white; the developing cell plate in light brown; and the cell wall in dark brown. **(a)** At late anaphase to telophase, MTs in the spindle midzone gradually appear in a bipolar array. Cross-linked MTs often show long overlapping zones. **(b)** At late telophase and cytokinesis onset, the phragmoplast MT array has a subpopulation of interdigitating MTs with a minimal overlapping zone and the rest of non-interdigitating MTs. **(c)** When the cell plate is assembled, the phragmoplast expands centrifugally as new mini-phragmoplast modules are added to the periphery and older ones are taken apart toward the center.

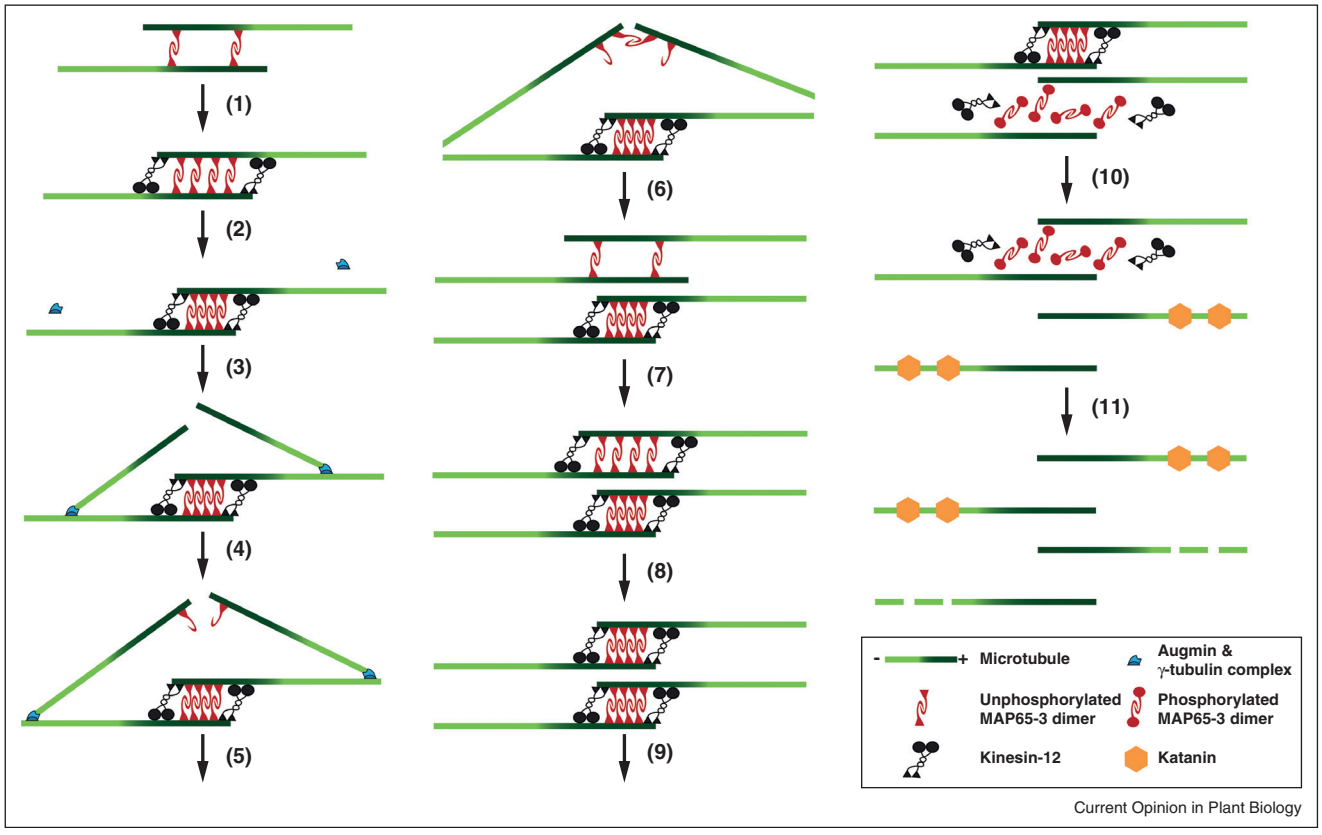
MTs are formed from coalesced MTs in the central spindle. At early stages during mini-phragmoplast development, Kinesin-12 is recruited to the plus ends of anti-parallel MTs in order to define the minimal MT-overlapping zone. Then the γ-tubulin complex is recruited to interdigitating MTs via the augmin complex to activate branched MT nucleation/polymerization preferentially toward the division plane. The newly polymerized anti-parallel MTs are captured by MAP65 toward their plus ends, followed by MT-induced multimerization of MAP65 to generate interdigitating MT bundles. While these events are taking place, additional non-interdigitating MTs are polymerized alongside the interdigitating MTs. When a mini-phragmoplast completes its mission, the MAP65 proteins associated with its MTs are phosphorylated by a MAP kinase pathway [14]. Consequently, MAP65 dimers are no longer in their multimeric form and dissociate from MTs. The resulting debundled MTs in the mini-phragmoplast are then disassembled via severing by enzymes like katanin and depolymerization by unknown MT depolymerases.

Anti-parallel MTs in the phragmoplast

The phragmoplast midzone is the destination of Golgi-derived vesicles and the site of cell plate assembly. The midzone is characterized by the meeting of the two halves of the phragmoplast MTs. Earlier observations by electron microscopy in the *Haemanthus* endosperm showed that these MTs are often interdigitated, associating with electron dense material [8]. Such a MT interdigitation phenomenon also has been shown in the moss *Physcomitrella patens* [17]. Although MT interdigitation was readily apparent in the endosperm phragmoplast of *A. thaliana* [18], the phenomenon was not obvious in its somatic cells undergoing cytokinesis [19]. Consistent with this, a dark midzone also can be seen in phragmoplast images of both anti-tubulin immunofluorescence in fixed cells and green fluorescent protein (GFP)-tubulin in live cells. Such a discrepancy could lead to the assumption that MT interdigitation is an ancient phenomenon in the phragmoplast which has been abandoned in somatic cells of higher plants. However, a number of MT-interacting factors like MAPs that bundle phragmoplast MTs, as well as the phragmoplast specific Kinesin-12 motors decorate the phragmoplast midzone exclusively and highlight bundle-like structures [20,21]. Thus it suggests that anti-parallel MT bundles have trespassed the phragmoplast midline, otherwise these proteins would not appear at that site.

Recent examinations of somatic cells by both electron and light microscopies have revealed that a small population of anti-parallel MTs interdigitate, while the majority do not cross the phragmoplast midline in *A. thaliana* [6••]. Interdigitation is mainly brought about by the MT cross-linker MAP65-3, a cytokinesis specific member of the evolutionarily conserved MAP65/Ase1 family including PRC1 in humans [6••,21]. Loss of MAP65-3 causes a widened phragmoplast midzone, likely due to the disappearance of interdigitating MTs, and frequent failures in cytokinesis [22]. Currently, it is unclear how the phragmoplast MT array maintains its bipolar appearance without MAP65-3. It is possible that scarce anti-parallel MTs are still cross-linked by other MAP65 proteins in the *map65-3* phragmoplast. In fact, three lines of evidence support that other MAP65 isoforms function redundantly with MAP65-3. A few

Fig. 2



Models depicting the amplification of the interdigitating MTs of the mini-phragmoplast module and their disassembly. Anti-parallel MTs are cross-linked by the MAP65-3 dimers first. The amplification process includes the following steps: (1) Multimerization of MAP65-3 dimer and recruitment of the Kinesin-12 motor; (2) Establishment of the minimal MT-overlapping zone to give rise to the core of the mini-phragmoplast module; (3) Recruitment of the augmin and γ-tubulin complexes for nucleation and polymerization of branched MTs; (4) MAP65-3 binding to unbundled anti-parallel MTs and outward sliding of interdigitated MTs; (5) Dimerization of MAP65-3; (6) Bundling of anti-parallel MTs; (7) As step (1); (8) As step (2). The disassembly of the MTs in the mini-phragmoplast requires the following steps: (9) Phosphorylation of MAP65-3 by the NACK-PQR pathway, followed by dissociation of MAP65-3 dimers from each other and from MTs; (10) Activation of katanin on debundled MTs; (11) Severing and disassembly of MTs in the mini-phragmoplast module.

other MAP65 isoforms decorate phragmoplast MTs with biases toward the midzone [21]. The loss of MAP65-1 or MAP65-2 enhances the growth defects in the *map65-3* null mutant [23]. Furthermore, ectopic expression of *MAP65-1* slightly rescues cytokinetic defects and the dwarfed growth phenotype in *map65-3* [24••]. However, the endogenous MAP65-1 protein does not highlight the phragmoplast midzone when examined by both immunofluorescence and GFP fusion either in the presence or absence of MAP65-3 [24••,25]. Thus the functional redundancy may lie in the fundamental aspects of MT dynamics like stability, instead of simply bundling MTs in the phragmoplast midzone.

While MAP65-3 conspicuously decorates the phragmoplast midzone, other MAP65 proteins exhibit different localization patterns [21]. So how do different MAP65 isoforms acquire different localization patterns in the phragmoplast? These proteins show the greatest variations in their C-terminal regions, which define their affinity for MTs and ability in regulating MT polymerization [21]. While the function of MAP65-3 in the phragmoplast cannot be replaced by MAP65-1, artificially grafting the C-terminal domain of MAP65-3 enables MAP65-1 to localize to the phragmoplast midzone and acquire most if not all the function of MAP65-3 [24••]. Thus the localization and function of different MAP65 isoforms are largely determined by their divergent C-termini, which may be achieved by interactions with other proteins in the phragmoplast and other arrays.

Another striking phenomenon is that proteins in the MAP65/Ase1 family specifically bundle anti-parallel MTs instead of parallel ones. *In vitro* experiments on two *Arabidopsis* MAP65 isoforms show that the proteins first bind to MTs in the monomeric form, and the dimerization process adds a zippering effect to cross-link anti-parallel MTs [26]. Moreover, it has been

demonstrated that MT-binding stimulates multimerization of Ase1, further recruiting the protein to overlapping MTs [27], a phenomenon that was also observed *in vitro* for MAP65-3 [6••]. As a result, the anti-parallel MTs are stabilized after being cross-linked by congregated MAP65-3. Besides engaging these MTs, these proteins function as platforms for other cytokinesis proteins such as kinesin motors, enabling specific actions on MTs in the phragmoplast midzone or the midbody in animal cells [6••,28].

A remaining puzzle stems from the fact that *in vitro* MAP65-3 tends to cross-link anti-parallel MTs extensively and sometimes across the entire length. However, *in vivo* the length of overlapped MTs is rather minimal in the phragmoplast [6••]. In vertebrates, the overlap length of PRC1-bundled anti-parallel MTs is controlled via overlap length-dependent microtubule growth inhibition by the Kinesin-4 motor, after it reaches the plus ends via its processive motility that is enhanced by a direct interaction with PRC1 [29•]. In plants like *A. thaliana*, Kinesin-4 motors have been assigned to regulate cell elongation in interphase [30]. Instead, the Kinesin-12 motors have been implicated in regulating phragmoplast integrity [31]. When two genes encoding functionally redundant Kinesin-12 motors are mutated, the phragmoplast MT array no longer forms a defined midzone [31]. MT polymerization continues at the plus ends after the anti-parallel MTs are cross-linked by MAP65-3 [32,33]. However, the plus ends of the interdigitating MTs have to be positioned at the phragmoplast midzone to define the destination of vesicle transport. Therefore, Kinesin-12 may regulate the overlapping length of the cross-linked MTs by sliding them apart when new segments are added to the plus ends (Figure 2).

It is noteworthy that in the moss *P. patens* MT interdigitation in the phragmoplast depends on the ungrouped/orphan kinesin KINID [17]. KINID is closely related to PAKRP2 in *A. thaliana*, which also specifically functions in the phragmoplast, but probably as a motor for vesicle transport [34]. In *P. patens*, there are three Kinesin-12 motors resembling those mentioned above [35]. Whether they function in a similar manner to their Arabidopsis counterparts remains to be tested. If so, it would be very interesting to learn if they function together with KINID to regulate extensive interdigitation of anti-parallel MTs in the moss phragmoplast.

Amplification of MTs at the phragmoplast periphery

The centrifugal expansion of the phragmoplast MT array requires MTs to be continuously amplified at the periphery. However, MT nucleation and polymerization take place along the entire phragmoplast, likely on existing MTs [36••]. Consistent with this, the MT-nucleating factor γ-tubulin decorates phragmoplast MTs with biases toward their minusends [37]. New MTs are preferentially polymerized toward the phragmoplast midzone and share the polarities of extant ones [36••]. This feature of shared polarities is similar to that seen for MT-dependent MT nucleation in interphase cells [38]. γ-Tubulin and the γ-tubulin complex proteins (GCPs) are associated with stable MTs that are aligned perpendicularly to the division plane in the phragmoplast [39••]. MTs nucleated by them exhibit dynamic instability and assume shallow angles to the division plane [39••]. Murata *et al*. found that newly polymerized anti-parallel MTs were preferentially cross-linked by MAP65 in the periphery of the expanding phragmoplast [39••]. The initial cross-linking could be joined by the recruitment of additional MAP65 dimers and then result in the stabilization of the anti-parallel MTs. The stable interdigitated MTs could serve as the core of a new module of the mini-phragmoplast being added to the periphery of the phragmoplast. The module becomes mature when additional MTs are polymerized on top of the interdigitated MTs.

Concomitant with continuous MT polymerization at the phragmoplast midzone, γ-tubulin displays a MT minus end-directed movement toward the distal ends of the phragmoplast, possibly via Kinesin-14 [39••]. Alternatively, this movement may be mediated by the translocation of the γ-tubulin-associated interdigitated MTs. The translocation is accompanied by MT polymerization at the plus ends. Consequently, MTs nucleated near the phragmoplast midzone would be able to reach similar lengths to those formed earlier.

The γ-tubulin complex typically binds to and caps MT minus ends, but cannot interact with MT lattices directly. Therefore, the association of the γ-tubulin complex on phragmoplast MTs must be mediated by protein(s) that directly interact with MTs. The 8-subunit complex augmin contains a MAP and serves as a docking factor for the γ-tubulin complex to localize to spindle MTs in animal and plant cells [40,41•,42••]. γ-Tubulin localization to phragmoplast MTs is largely dependent on augmin as it becomes dissociated from the phragmoplast in augmin mutant cells in *A. thaliana* [42••]. In the mutant, the phragmoplast MT array often fails to expand toward the cell periphery and MTs ultimately became disorganized [42••]. Similarly when genes encoding augmin subunits are silenced in the moss *P. patens*, phragmoplast expansion is inhibited because of compromised MT formation in the array [43••]. Thus, the MT-dependent MT nucleation mechanism, mediated by the augmin and γ-tubulin complexes, is shared in both the spindle and phragmoplast for amplifying MTs.

Phragmoplast MT turnover

New MTs are polymerized at the phragmoplast periphery and as soon as cell plate assembly is underway, older ones

toward the center are disassembled. The addition of new MTs is at the expense of depolymerization of old ones because taxol-mediated inhibition of MT depolymerization blocks phragmoplast expansion [44]. When MTs are bundled, they become resistant to depolymerization challenges. The mitogen-activated protein kinase (MAPK) cascade, including the NPK, NQK, and NRK (PQR) kinases, is deployed to initiate MT turnover [14]. The MAPK cascade is activated by the Kinesin-7 NACK1 in tobacco or HINKEL in *A. thaliana* in the phragmoplast midzone, and the terminal kinase of the cascade phosphorylates MAP65 family proteins including MAP65-1, -2, and -3 [23,45]. Consequently, phosphorylated MAP65 shows greatly reduced MT-bundling activity when compared to unphosphorylated one [45]. Conversely, the non-phosphorylatable MAP65 mutant protein has enhanced MT-bundling activity [45]. Although multiple MAP65 isoforms can be phosphorylated, MAP65-3 shares a similar localization pattern as HINKEL/NACK and the PQR kinases in the phragmoplast [6••]. In contrast, MAP65-1 and MAP65-2 localize along phragmoplast MTs when they are expressed as GFP fusions at physiologically relevant levels in *A. thaliana* [24••,25]. So MAP65-3 may be a dominant substrate of the MAP kinase of the NACK-PQR pathway in the phragmoplast in *A. thaliana*. It is possible that MAP65-3 can multimerize and bundle anti-parallel MTs toward their plus ends when it is not phosphorylated by the NACK-PQR pathway. The phosphorylation may prevent the MAP65-3 dimers from forming multimers and cause their dissociation from MTs in the phragmoplast midzone.

When MTs are no longer bundled in the phragmoplast, single MTs may be translocated along neighboring parallel MTs toward their minus ends for depolymerization. In fact, this MT translocation event can be artificially enhanced in glycerol-permeabilized cells undergoing cytokinesis that is accompanied by the accumulation of kinesin motors toward the distal edges when exogenous ATP is provided [46]. In addition to MT translocation, however, other mechanisms must be accountable for the disassembly of phragmoplast MTs. At late stages of cytokinesis, the phragmoplast MT arrays tend to appear in concave configurations with shorter MTs toward the center of the phragmoplast. This would suggest that the primary site of MT depolymerization is at the distal minus ends. A potential factor that may contribute to MT disassembly is the severing factor katanin (KTN) because the KTN1/p60 subunit decorates phragmoplast MTs [47]. If the minus ends of phragmoplast MTs are stabilized by anchoring to cellular structures, katanin would sever MTs and release these MTs from the anchorage. Whether there are separate MT depolymerases that function in the depolymerization of phragmoplast MTs, like Kinesin-13 on kinetochore fiber MTs in animal cells, remains to be determined.

It is noteworthy that inhibition of vesicle trafficking or membrane remodeling, for example, treatment with Brefeldin A or caffeine, inhibits MT depolymerization and block the expansion of the phragmoplast MT array [48,49]. Because caffeine likely blocks membrane reorganization after the fusion of Golgi-derived vesicles in the phragmoplast [50], MT depolymerization is likely induced at late stages of cell plate assembly. It would be interesting to examine whether membrane reorganization releases or activates certain MT depolymerization factors during cell plate assembly.

Conclusions and perspectives

The modular model discussed here serves as a gateway for future investigations of the assembly and development of the phragmoplast MT array. A number of critical questions immediately arise in regard to specific activities of critical factors and the coordination of different events in the phragmoplast. Among them, how is Kinesin-12 recruited to the phragmoplast midzone? Why does MAP65-3 only bundle MTs toward their plus ends? How do MAP65-3 and Kinesin-12 coordinate to establish the narrow MT-overlapping zone in the phragmoplast? During cell plate assembly, how is membrane remodeling coordinated with MT disassembly? What is the MT depolymerization factor that contributes to the disassembly of phragmoplast MTs?

In addition to the aforementioned factors, the Fused family kinase TIO (Two-In-One), initially identified as a critical factor in male gametogenesis, localizes to the phragmoplast midzone in somatic cells as well and is required for cytokinesis [51]. It interacts with Kinesin-12 in a yeast two-hybrid assay [52]. It would be interesting to test whether the function of the cytokinesis specific motor is regulated by TIO. Another kinase-like MAP RUNKEL also decorates the phragmoplast midzone and is required for the organization of the anti-parallel MT array and the expansion of the phragmoplast [53,54]. It will be fascinating to elucidate how so many cytokinesis important factors work together in a spatially and temporally coordinated manner to govern the rise and fall of the phragmoplast MT array.

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