Distinctive Kinesin-14 Motors Associate with Midzone Microtubules to Construct Mitotic Spindles with Two Convergent Poles in Arabidopsis

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Abstract

Microtubule (MT) motors in the Kinesin-14 subfamily proliferated in photosynthetic organisms and they often incorporated sequences bearing novel structural features. To gain insights into the functions of diversified Kinesin-14 motors from an evolutionary perspective, we performed phylogenetic analyses across different eukaryotic kingdoms. Compared to fungi that have a single class of Kinesin-14, the early divergent protist Giardia possesses two classes and the motile green alga Chlamydomonas produces four classes (Kinesin-14A to Kinesin-14D). The fifth class Kinesin-14E first appeared among immotile green algae and the sixth Kinesin-14F emerged in mosses, concomitantly with the display of 3D growth. The conservation of Kinesin-14D from green algae prompted us to investigate its function in Arabidopsis in which three such motors functioned in cell cycle-dependent manners. They localized on selective spindle MTs and/or sometimes kinetochore-like structures, and later all became conspicuous on MT bundles in the spindle midzone following sister chromatid segregation. Genetic dissection of Kinesin-14D1 showed that its loss led to hypersensitivity to low doses of the MT-depolymerizing herbicide oryzalin. Kinesin-14D1 association with the midzone MTs in both prophase and mitotic spindles. The oryzalin treatment left behind discrete kinetochore fibers attached to randomly positioned chromosomes in the mitotic kinesin-14d1 cells but prevented the pole convergence of bipolar mitotic spindles. This function of Kinesin-14D1 in the spindle midzone is likely dependent on an MT-binding domain at the C-terminus to the catalytic motor domain. Therefore, our results revealed a novel Kinesin-14D-dependent mechanism that regulates the formation of bipolar spindle apparatus with converged acentrosomal poles.

Introduction

Microtubules (MTs) are polar polymers that function as tracks for motor-driven directional transport inside eukaryotic cells. There are two classes of MT-based motor proteins, namely dyneins and kinesins that hydrolyze ATP and convert the chemical energy to mechanical actions along MTs \(^1\). While dyneins are MT minus end-directed motors, kinesins include both plus and minus end-directed motors as well as ones that function as MT depolymerases at MT ends instead of powering directional motility \(^1,\ 2\). Compared to dyneins, the kinesin superfamily proteins have more diversified forms and can be divided into 14 or more subfamilies based on the sequence homology in the catalytic domains of the polypeptides \(^3-5\). Among them, the Kinesin-14 subfamily was thought to have two classes and these motors generally are considered MT minus end-directed motors in contrast to most kinesins like the conventional kinesin or Kinesin-1 that travel towards MT plus end \(^4\). Genome sequences have indicated that the Kinesin-14 subfamily is more complex than thought initially.

The founding members of the Kinesin-14 subfamily are KAR3 (Karyogamy) in budding yeast and Ncd (Nonglareted disjuncional) in flies \(^6-8\). Unlike conventional kinesin and most MT plus end-directed kinesins that have the catalytic domain with ATP- and MT-binding sites located at the N-terminal side of the polypeptides, KAR3 and Ncd have it at the C-terminus. In contrast to fungi that often make only one
Kinesin-14 motor for a given species, plants produce a greater number of different motors in this subfamily. For example, there are 21 genes encoding Kinesin-14s in the model plant Arabidopsis thaliana. Earlier sequence analysis revealed the explosive increase of Kinesin-14 genes in photosynthetic organisms. In angiosperms like A. thaliana and rice (Oryza sativa), Kinesin-14 can be grouped into six classes that have their catalytic domains at the N-terminus, C-terminus, or center of the polypeptides. Similarly, the moss Physcomitrella patens and the fern Marsilea vestita already have such six classes of Kinesin-14. In the literature, it has been speculated that flowering plants produce so many different types of Kinesin-14 motors because they, unlike protists, fungi, and animals, do not possess genes encoding dyneins that act as MT minus end-directed motors.

Following the initial isolation of Kinesin-14 genes encoding Kat or kinesin-like protein in A. thaliana, we have only learned the functions of a few of these motors. In A. thaliana, the loss of the zwi (zwischel) gene encoding KCBP (Kinesin-like Calmodulin-Binding Protein), a Kinesin-14 with a calmodulin-binding site and a myosin tail homology domain, leads to reduced branching in unicellular leaf trichomes. However, KCBP associates with all MT arrays with particular emphasis at the spindle poles during mitosis in flowering plants, implying its function in cell division as well. The atk1-1 mutation in the KatA gene causes defects in spindle MT organization with split spindle poles during male meiosis and consequently male sterility. This motor is detected on spindle and phragmoplast MTs and plays a role in spindle morphogenesis in mitosis as well. A class of Kinesin-14 with the Calponin Homology (CH) domain, KCH interacts with actin microfilaments. Different KCH kinesins have been detected along cytoplasmic MTs in tip-growing protonemal cells in P. patens, the preprophase band (PPB) at the cell division site in rice, and various MT arrays during cell cycle in tobacco cells so that their functions are interpreted like cytoskeletal coalescence for tip growth, cell division plane determination, and cell cycle progression. Although these findings have provided some clues on the functional diversification of Kinesin-14 in plants, we remain puzzled by questions like why the Kinesin-14 subfamily expands so dramatically with serious increases in gene numbers in many classes and how these different Kinesin-14 classes have emerged.

The primitive divergent eukaryote Giardia lamblia has 25 kinesin genes among which two are Kinesin-14s with one resembling KAR3/Ncd type. In the unicellular green alga Chlamydomonas reinhardtii, among 23 kinesins five belong to the Kinesin-14 subfamily and can be aligned with ones from plants in four different classes. Therefore, the LECA (Last Eukaryote Common Ancestor) perhaps already had multiple forms of Kinesin-14 motors that exercise different functions as MT minus end-directed motors. During the evolution towards angiosperms, these different Kinesin-14 motors are inherited while new classes might have emerged. Here, we performed a comprehensive phylogenetic analysis of the Kinesin-14 motors encoded by recently sequenced genomes of representative green photosynthetic organisms from green algae to land plants and revealed when the six classes of Kinesin-14 motors first appeared in the context of growth complexity. Furthermore, we found that motors of the Kinesin-4D class selectively associated with MTs in the spindle midzone during mitosis in A. thaliana and specifically, Kinesin-14D1.
played a novel, non-redundant role in the organization of kinetochore fibers into the convergent apparatus of bipolar spindles.

**Results**

**The emergence of 6 classes of Kinesin-14 motors in land plants**

To gain a comparative view of Kinesin-14 motors in photosynthetic organisms, we included Kinesin-14 polypeptides encoded by genomes of representative organisms that represent significant advancements towards angiosperms. Green algae include *C. reinhardtii* which is motile, unicellular organism, the charophyte *Klebsormidium nitens* which becomes immobile and shows multicellular filamentous growth, and *Chara braunii* which is one of the earliest that produce the cytokinetic apparatus of the phragmoplast \(^{30,31}\). Two bryophytes, the liverwort *Marchantia polymorpha* and the moss *P. patens*, represent basal and advanced non-vascular land plants, respectively \(^{13,32}\). The lycophyte *Selaginella moellendorffii* was chosen to represent early vascular plants \(^{33}\). We included the kinesins from the basal eukaryote *G. lamblia*, the filamentous fungus *Aspergillus nidulans*, and the vertebrate mouse (*Mus musculus*) to reveal the evolutionary relationship. The catalytic or motor domains of Kinesin-14s from these organisms were compared to those of the six classes of Kinesin-14s from two angiosperms, monocotyledous rice (*O. sativa*) and dicotyledous *A. thaliana* \(^{11}\).

We classified Kinesin-14 motors into class A to F by correlating their initial appearance in these representative photosynthetic organisms with the hierarchical order of these organisms (Figure 1A, B, Supplemental Table). Class A Kinesin-14 is represented by Kar3/Ncd; class B is KCBP-type; class C is the KCH-type; class D includes motors with the malectin domain \(^{34}\); class E is the KCA or KAC-kind of motors; and class F is the most recent one. The KAR3/Ncd-type kinesis-14 motors are conserved among all eukaryotes. When only a single Kinesin-14 motor is preserved as seen in many fungal species, it belongs to this class, like KLPA in *A. nidulans* \(^{35}\). We found that in *G. lamblia* one Kinesin-14 belongs to KAR3/Ncd-type and another one is most closely related to KCBP (Figure 1 and Supplemental Figure 1). Therefore, we conclude that KCBP, similar to Kinesin-14A (KAR3/Ncd-like), belongs to an ancient type of kinesin. The five kinesins in *C. reinhardtii* are in four classes (Kinesin-14A to Kinesin-14D). Compared to *G. lamblia*, this green alga has added Kinesin-14C (KCH) and Kinesin-14D motors.

In contrast to *C. reinhardtii*, the multicellular green alga *K. nitens* has a gene encoding the fifth class Kinesin-14, the KCA/KAC motor, coincidently with the transition from having mobile vegetative cells to becoming immobile during vegetative growth (Figure 1). Such motors are implicated in chloroplast distribution in *A. thaliana* \(^{36}\). Similarly, the more advanced green alga *C. braunii* and the bryophyte *M. polymorpha*, a liverwort, also have these five classes of Kinesin-14 and a single form in each class (Figure 1A, B). However, the more sophisticated bryophyte *P. patens*, a moss, has added the sixth class of Kinesin-14. Because the moss marks the transition from producing flat thalli (2D) to those with stem-like structures (3D) during vegetative growth in land plants \(^{37}\), the addition of this Kinesin-14E class may be
associated with this evolutionary advancement. Following the advanced bryophytes like mosses, all vascular plants contain these six classes of Kinesin-14 (Figure 1A, B).

In contrast to classes A, B, C, and E that have been studied by both genetic and cell biological means for their distinct cellular and developmental function in photosynthetic organisms, limited information has been obtained for the motors in the other two classes. While the class Kinesin-14F may function in the context of 3D growth, here we focus our functional analysis on Kinesin-14D motors in *A. thaliana* specifically because of their unique structural features (Figure 1C) and elevated expressions in dividing cells.

**Kinesin-14D1 associates with midzone MTs but not kinetochore fibers**

Because class D Kinesin-14 motors are present from motile unicellular green algae to land plants, we hypothesized that they likely possess unknown functions that are shared among the organisms. The three motors in this class in *A. thaliana*, namely Kinesin-14D1 (previously known as MDKIN2) encoded by the At2g22610 locus, Kinesin-14D2 (or MDKIN1) by At1g72250, and Kinesin-14D3 by At5g27550 were previously determined to be mitotic kinesins that exhibit cell cycle-dependent expression patterns. All three kinesins have the catalytic domain located in the middle of the polypeptides that are preceded by the typical Kinesin-14 type neck linker (Figure 1D). The catalytic domains are followed by novel C-terminal domains containing predicted coiled-coils. Furthermore, Kinesin-14D1 and Kinesin-14D2 contain a malectin domain towards the N-terminus as revealed recently. To determine their localizations and functions, we first produced constructs that would have the motors expressed in fusion with green fluorescent protein (GFP) under the control of their native promoters in *A. thaliana*. Because these genes were transcriptionally detected as mitotically active, we employed the inducible mitotic CDELS system in tobacco leaves to detect their activities in dividing cells that recapitulate their native conditions. When the leaf epidermal cells were induced to enter mitotic cell division, we found that the Kinesin-14D1-GFP fusion protein underwent drastic relocalization from the nucleoplasm at early stages of prophase indicated by conspicuous PPB (Figure 2A, B). The signal was excluded from the condensed chromosomes reported by histone H1 tagged with the TagRFP fluorescent protein (Figure 2A). When cells entered prometaphase following PPB disassembly, the Kinesin-14D1-GFP signal was detected on MT bundles and was particularly pronounced on MT bundles in the middle of the prophase spindle but not on more prominent MTs toward two polar regions (Figure 2B). When mitosis progressed to metaphase, Kinesin-14D1-GFP remained in the spindle midzone as if it highlighted MT bundles running between chromosomes aligned at the metaphase plate (Figure 2A). The GFP signal did not overlap with prominent kinetochore fibers (Figure 2B). At anaphase when sister chromatids were segregated to two poles, the Kinesin-14D1-GFP fusion protein remained in the midway between the two groups of chromatids (Figure 2A). At this stage, the kinetochore fibers were shortening while fine MT filaments were polymerized between the shortened fibers (Figure 2B). Kinesin-14D1-GFP exhibited a distribution pattern as if it marked find MT bundles there (Figure 2B). To discern whether the motor colocalized with MT...
bundles formed between segregated sister chromatids, we compared the two signals in an enlarged view and found that Kinesin-14D1 did not overlap with the majority of obvious MT bundles but rather flanked them, as further revealed by fluorescence intensity scans (Figure 2C). Occasionally, there were a few detectable MT bundles had the kinesin signal overlapped. At telophase and cytokinesis when cells were reforming daughter nuclei, the Kinesin-14D1-GFP signal became more concentrated towards and restricted to the division site (Figure 2A). The motor occupied the midline of the phragmoplast MT array during cytokinesis (Figure 2B). Although a recent report showed a similar Kinesin-14D1/MdKin2-GFP in the spindle midzone and phragmoplast midzone, our findings brought novel insights into the featured localization pattern inside the spindle apparatus.

**Kinesin-14D2 and Kinesin-14D3 are associated with kinetochores and spindle midzone**

To examine their cellular localizations, similar GFP fusion proteins of Kinesin-14D2 and Kinesin-14D3 were expressed in the CDELS under the control of their native promoters. The Kinesin-14D2-GFP fusion protein was barely detectable prior to the nuclear envelope breakdown at prophase (Figure 3A, B). At prometaphase, however, the motor labeled spindle-like structures surrounding condensing chromosomes (Figure 3A). The signal mostly overlapped with spindle MTs with clear emphasis towards spindle poles (Figure 3B). When the chromosomes were aligned at the metaphase plate, the Kinesin-14D2-GFP fusion protein had kinetochore fiber-like localization as well as on kinetochores (Figure 3A, C). The colocalization with kinetochore fibers and kinetochores were confirmed with MTs were reported by mCherry-TUB6 (Figure 3B, C). As anaphase progressed, the Kinesin-14D2 signal remained in the middle of the cell, between two segregating groups of sister chromatids, and it became more and more prominent with a subpopulation of fine MT bundles in the spindle midzone (Figure 3A, B). When cells entered cytokinesis as the daughter nuclei started to form and a bipolar phragmoplast MT array emerged, Kinesin-14D2-GFP became concentrated in the phragmoplast midline (Figure 3A, B).

Because Kinesin-14D1 and Kinesin-14D2 exhibited different mitotic localization patterns despite bearing similar functional domains, we then asked where the third motor in this class, Kinesin-14D3 localized differently during mitosis. Unlike the other two, Kinesin-14D3 lacks the N-terminal malectin domain. The Kinesin-14D3-GFP fusion protein, expressed under the control of the *Kinesin-14D3* promoter, was first detected on the prophase nuclear envelope where rich MTs were nucleated (Figure 4A, B). After the nuclear envelope breakdown, it filled the space between condensing chromosomes (Figure 4A). The fusion protein became concentrated in the middle of the developing spindle as if it defined its midzone (Figure 4B). In metaphase cells, Kinesin-14D3-GFP was mostly concentrated on paired kinetochores with additional signals trailing the kinetochores in the spindle midzone (Figure 4A, B). During anaphase, the motor increasingly emerged between segregated sister chromatids, highlighting MT bundles in the spindle midzone, while the kinetochore localization was still detected (Figure 4A-C). When sister chromatids reached two poles, Kinesin-14D3-GFP was no longer detected at the kinetochores while its association with the midzone MTs of the spindle midzone became even more striking (Figure 4A, B). This
midzone localization persisted and became more and more concentrated towards and restricted to the division site and highlighted the phragmoplast midzone throughout cytokinesis (Figure 4A, B). Therefore, the three Kinesin-14D motors exhibited distinct dynamic localization patterns while sharing the emphasized association with the spindle midzone and the phragmoplast midline that distinguished them from other Kinesin-14 motors and those in other subfamilies in *A. thaliana*.

**The loss of Kinesin-14D1 leads to hypersensitivity to MT depolymerization challenges**

The cell cycle-dependent localization patterns of these motors prompted us to test their functions by employing T-DNA insertional mutants. We isolated homozygous mutants of the *kinesin-14d1* mutation with a T-DNA insertion in the 11th exon of the *Kinesin-14D1* locus; *kinesin-14d2-1* and *kinesin-14d2-2* with insertions in the 11th and 13th introns, respectively, of the *Kinesin-14D2* locus; and *kinesin-14d3* in the 4th intron of the *Kinesin-14D3* locus. These mutants grew indistinguishably from the wild-type control as demonstrated by seedling growth on agar media (Figure 5A). When they were challenged by the MT-depolymerizing herbicide oryzalin at 100 nM, however, the *kinesin-14d1* seedlings no longer produced viable roots (Figure 5B). Such a challenge did not noticeably alter growth of the wild-type seedlings (Figure 5B). The oryzalin-inhibited growth phenotype in the *kinesin-14d1* mutant was completely suppressed by the Kinesin-14D1-GFP fusion, as described above, expressed under the control of its native promoter (Figure 5B). This result informed us that not only the oryzalin hypersensitivity was linked to the *kinesin-14d1* mutation but also the Kinesin-14D1-GFP fusion protein was functional. Therefore, we concluded that Kinesin-14D1 bears some unique functions that are not shared by the other two members of motors in this class, that is consistent with its dynamic localization during mitosis that distinguished from those of the other two.

**Kinesin-14D1 plays a critical role in the integration of the kinetochore fiber complexes into a bipolar spindle**

Because an oryzalin hypersensitivity phenotype could be linked to defective MT activities, we first tested whether the *kinesin-14d1* mutant cells exhibited abnormal MT arrays by live-cell imaging of mutant cells expressing a GFP-TUB6 fusion protein. While the cortical MT arrays, the PPB, and the perinuclear spindle arrays at prophase did not exhibit noticeable abnormalities, the spindle MT arrays following nuclear envelope breakdown were seriously altered (Figure 5C). Instead of forming a fusiform array, kinetochore fibers were aligned in parallel as if they lacked the guidance to be focused (Figure 5C). Such a fence-like pattern persisted during anaphase although all kinetochore fiber MTs shortened concomitantly (Figure 5C and Supplemental Movie 1). Such morphologic defects were corrected when the Kinesin-14D1-GFP fusion protein was expressed (Figure 5C and Supplemental Movie 2). Following the completion of karyokinesis, MTs were successfully assembled into the phragmoplast array that was similar to ones formed in the cell expressing a Kinesin-14D1-GFP (Figure 5C).
To quantify how spindle organization was affected in the mutant, we measured the widths of spindle poles and spindle midzone in both the mutant and the control plant after their cells were fixed for anti-tubulin immunofluorescence staining. The ratio of pole width to midzone width significantly increased in the mutant cells (Figure 5D). Therefore, we concluded that the collective convergence of the kinetochore fibers was most noticeably affected upon the loss of Kinesin-14D1.

To test how low doses of oryzalin caused seedling lethality and whether the lethality was brought about by an enhanced defects in spindle MT organization during mitosis, we examined mitotic MT arrays after the oryzalin challenge in the kinesin-14d1 mutant expressing GFP-TUB6. The mutant cells had kinetochore fibers formed but failed to be integrated into an organized spindle array that was formed in the control cells under identical conditions (Figure 5E and Supplemental Movie 3 and 4). These kinetochore fibers remained disorganized for over 30 min after nuclear envelope breakdown while the rescued cells expressing Kinesin-14D1-GFP already completed anaphase as indicated by kinetochore fiber shortening within 30 min, indicating that anaphase onset was seriously delayed in the kinesin-14d1 mutant. Therefore, we hypothesized that the oryzalin treatment arrested mitosis at prometaphase in the mutant cells so that the root could no longer produce new cells.

To test this hypothesis and quantify the mitotic defect brought about by the loss of Kinesin-14D1, we examined MT organization in the oryzalin-treated mutant cells by immunofluorescence microscopy. Upon the disassembly of the PPB, rich peri-nuclear MTs were formed around the time of nuclear envelope breakdown (Figure 5F). Defects were most pronounced after nuclear envelope breakdown in the mutant cells. Instead of having kinetochore fibers organized and bipolarized as in the control cells, the mutant cells formed kinetochore fibers were placed separately from each other and not integrated into a spindle configuration in oryzalin-treated mutant cells (Figure 5F). Concomitantly, chromosomes were not perfectly aligned at the metaphase plate in cells containing condensed chromosomes (Figure 5F). These phenotypes were suppressed when Kinesin-14D1 was expressed under its native promoter (Figure 5F). Taken together, we concluded that the oryzalin treatment probably triggered spindle assembly checkpoint in the mutant cells so that they eventually lost viability due to mitotic arrest.

**Functional domains of Kinesin-14D1 render distinct localization patterns upon ectopic expression**

Kinesin-14D1 possesses novel domains outside the conserved catalytic domain including an N-terminal malectin domain as revealed previously. Because the localization pattern of kinesin motors largely is dependent on the nonmotor domains, we tested their potential contributions to Kinesin-14D1’s cell cycle dependent activities using different truncations (Figure 6A). When the Kinesin-14D1 Dmotor fragment derived by removing the motor domain was expressed in fusion with GFP under the control of its native promoter, the fusion protein first was in the nucleus and then decorated the spindle and phragmoplast MT arrays uniformly following nuclear envelope breakdown (Figure 6B). Such a localization pattern might be attributed to the presence of an MT-binding site outside the motor domain. To test this
possibility, we then took this Kinesin-14D1\textsuperscript{Dmotor} truncated protein apart into smaller fragments and again expressed in GFP fusions under the native promoter. Because the malectin domain often is said to be carbohydrate-binding, we first took this domain and examined the GFP fusion of this N-terminal Kinesin-14D1\textsuperscript{N} fragment. The fusion protein was concentrated near both spindle and phragmoplast MT arrays but unlike MT bundles appeared more in a uniform pattern within the arrays (Figure 6C). When the entire fragment following the motor domain was expressed, the GFP-Kinesin-14D1\textsuperscript{c} fusion protein decorated both spindle and phragmoplast MTs as well as the chromatin (Figure 6D). This dual localization pattern prompted us to test whether the smaller C-terminal fragment following the coiled-coil domain contributed specifically to one of the two sites. We found that Kinesin-14D1\textsuperscript{c'-GFP} was in the prophase nucleus and associated with mitotic chromosomes (Figure 6E). Therefore, we concluded that the C-terminal domains enabled the motor’s interaction with chromatin and MTs in the mitotic arrays.

Kinesin-14D1 decorates a selective group of spindle MTs in the spindle midzone

To test the hypothesis of MAP65-independent MT bundling by Kinesin-14D1 in the spindle midzone, we took one step further to examine its localization with centromeres as the reference first. Kinesin-14D1\textsuperscript{mCherry} was abundantly detected in the spindle midzone when paired centromeres, marked by a GFP-HTR12/CENH3/CENPA fusion protein, were clearly detected (Figure 7A). However, discrete Kinesin-14D1-highlighted regions often were displaced from the centromeres, particularly noticeable towards the periphery (Figure 7A). In Arabidopsis thaliana, MAP65-4 is abundantly detected on MTs towards the spindle midzone including kinetochore fibers. So, we examined its localization using a MAP65-4-mCherry fusion protein co-expressed with GFP-HTR12 in mitotic tobacco cells (Figure 7B). Centromeres landed on MAP65-4-highlighted MT bundles in merged images (Figure 7B). Therefore, the result suggested that Kinesin-14D1 and MAP65-4 perhaps decorated different populations of anti-parallel MTs in the spindle midzone. To test this hypothesis, we co-expressed Kinesin-14D1-GFP and MAP65-4-mCherry fusion proteins and compared their localizations. Although both proteins marked prominent MT bundles in the spindle midzone, their localizations differed in several aspects. First, Kinesin-14D1 exhibited a wider distribution than MAP65-4 pattern transversely in the division plane. But MAP65-4 had a wider distribution pattern than Kinesin-14D1 vertically in the spindle axis. Furthermore, the signal strength as revealed by fluorescence intensity scan showed the peaks in two channels did not always overlap and MAP65-4 was more concentrated towards the center and Kinesin-14D1 had strong peaks towards the periphery (Figure 7C). Such differences became more pronounced when the images were visualized after binarization that enhanced the discrepancy of the localizations (Figure 7C). Because Kinesin-14D1-GFP signal did not overlap with prominent MT bundles in the spindle midzone at anaphase (Figure 2C), we then asked whether MAP65-4 decorated these bundles by co-expressing MAP65-4-mCherry fusion protein and GFP-TUB6 (Figure 7D). In the cell with prominent MT bundles in the spindle midzone, the MAP65-4 signal overlapped that of MTs, which was further indicated by fluorescence intensity scan (Figure 8D). Therefore, we concluded that anti-parallel MT bundles in the spindle midzone could be divided into at
least two populations, the prominent one with MTs bundled by MAP65 and the less prominent one with MTs cross-linked by Kinesin-14D1.

**Discussion**

Kinesin-14 motors often are known for their functions in clustering the minus ends of parallel MTs for the organization of acentrosomal spindle poles. Our results from the interrogation of Kinesin-14 during plant evolution revealed that in contrast to the Kinesin-14A class fulfilling this MT minus end-targeted roles, the motors in the Kinesin-14D class acted on anti-parallel MTs in the midzone of spindles, perhaps MT plus ends emphasized, and Kinesin-14D1, as the first protein detected in the midzone of prophase spindles, played a critical role in organizing paired kinetochore fibers into an orchestrated bipolar spindle. The findings challenged the existing view of simplified mitotic function of Kinesin-14 and uncovered a novel mechanism of spindle organization based on our results summarized here (Figure 9).

**Kinesin-14D is an ancient class of Kinesin-14**

Kinesin-14 is made in most eukaryotes except Apicomplexa species. Early phylogenetic analysis suggests that there are two classes of Kinesin-14 motors and has those plant members divergent from the class I (represented by ATK1/KatA) grouped into the second class, together with KIFC2 and KIFC3 from mammals. Based on the sequence alignment of the motor domain, our phylogenetic analysis showed that the green alga *C. reinhardtii* clearly has four classes of Kinesin-14 including Kinesin-14D that are present in all green algae and land plants. Therefore, these Kinesin-14D motors not only are present in all land plants as reported earlier, their deep evolutionary roots go back to green algae although it is unclear whether the algal Kinesin-14D exercises similar mitotic functions as reported here.

In contrast to class A Kinesin-14 which has a nucleotide-independent MT binding site at the N-terminus followed by coiled-coil domains leading the C-termina motor domain, the three Kinesin-14D motors described here all have the motor domain located in the middle of the polypeptides. However, they all possess the typical neck peptide shared by other reported Kinesin-14 motors at the N-terminal side of the motor domain. Interestingly, Kinesin-14D1 and Kinesin-14D2 distinguish from other kinesins for possessing a malectin homology domain at the N-terminal non-motor region. Malectin is a carbohydrate-binding protein known for its role in protein N-glycosylation in the endoplasmic reticulum. In flowering plants, the malectin homology domain is mostly noticed in malectin-like receptor kinases including Feronia (FER) in *A. thaliana* and is recognized to constitute the extracellular carbohydrate ligand-binding site for receptor activation. Therefore, its presence in a kinesin is surprising because the motor's mission is restricted within the cytoplasm. When it was taken out of Kinesin-14D1 and expressed in a GFP fusion, the fusion protein exhibited an uncharacteristic localization pattern, although being enriched near the spindle apparatus. It would be interesting to learn the functionality of this domain.
Like the Kinesin-14 motors in class A including ATK1 and ATK5, all three Kinesin-14D motors are considered mitotic kinesins based on their cell cycle-dependent expression patterns\(^3\). Both the imaging data and the mitotic phenotype linked to the loss of function mutation were consistent with the transcription-based prediction. This raises the question of whether motors belonging to this class in also function in mitosis in other organisms. To date, only the mouse KIFC3 has been learned to function in centrosome cohesion at the mitotic onset to prevent premature centrosome separation, prior to the action of the MT plus end-directed, Kinesin-5 motor Eg5 that separates the centrosomes at prophase\(^4\). In \textit{A. thaliana}, a different scenario was determined here that distinguishes Kinesin-14D1 from those previously reported ones in at least two aspects. First, Kinesin-14D1 resided in the nucleus prior to nuclear envelope breakdown. Second, it specifically associated with MTs in the spindle midzone as soon as the nuclear envelope broke down. Such a continuous appearance in the designated region of special group of spindle MTs probably was brought about by the novel domains found in Kinesin-14D1, for example.

Although bearing a typical Kinesin-14 neck sequence for MT minus end-directed motility, Kinesin-14D1 exhibited a localization pattern that suggested that it might act like a plus end-directed motor or hitchhiked on a plus end-directed motor like Kinesin-5 because it accumulated in regions where MT plus ends were clustered. To further determine Kinesin-14D1 motility, \textit{in vitro} motility assays may be employed. Using purified Kinesin-14D1 fusion proteins, however, we have not been able to demonstrate its motility in vitro (data now shown). Alternatively, rigor mutant form of Kinesin-14D1 may be generated and a Kinesin-14D1 (rigor)-GFP fusion protein is expressed in cells bearing spindles. If it were a plus end-directed motor, we would expect the rigor mutant to be dissipated from the spindle midzone and perhaps relocated to the spindle poles. A minus end motor would do the opposite.

**Kinesin-14D1 organizes chromosome/kinetochore-independent MTs in acentrosomal spindles in \textit{A. thaliana}**

The mitosis specific expression of Kinesin-14D motors was consistent with their association with the mitotic apparatus. Concomitant with nuclear envelope breakdown, Kinesin-14D1 selectively appeared in the midzone of the developing prophase spindle, illustrating the bipolar feature. The prophase spindle often has highly focused poles, also referred to as the MT-converging center, prior to nuclear envelope breakdown as clearly demonstrated in the elegant mitotic system of \textit{Haemanthus} endosperm\(^5\). This feature of prophase spindles with highly focused poles is observed in plant spindles after the loss of the centrosome and may be linked to the orientation of the mitotic spindle following the establishment of the kinetochore fibers\(^6\). When the function of factors that are fundamentally important for MT assembly of organization, like the \(g\)-tubulin complex protein GCP6, is compromised, not only the prophase spindle is compromised but also the later mitotic spindle becomes disorganized\(^7\). Hence, it would be ideal to specifically disturb the non-kinetochore fiber MTs in order to learn how the prophase spindle may contribute to organizing a mitotic spindle with two converged poles. Results summarized here showed that the Kinesin-14D-dependent bipolar prophase spindle constitutes a mechanism that engages
kinetochore bers established after the nuclear envelope breakdown. Our results indicate that motors like Kinesin-14D1, and perhaps other two isoforms as well, likely contribute to the bipolarization of the prophase spindle (Figure 9A). Such bipolar prophase spindle sets up the status quo of the mitotic apparatus upon the breakdown of the nuclear envelope when chromosomes/kinetochores start to nucleate new MTs or capture MTs nucleated elsewhere (Figure 9B). In other words, the prophase spindle MTs, although being dynamic, provide a framework for anchoring developing kinetochore bers, as suggested before 49. When the kinetochore bers are fully established, they are integrated with the prophase spindle to give rise to the mitotic spindle with two converged poles informed by the prophase spindle (Figure 9C). We hypothesize that such a scenario is not reserved in plant cells and may be shared by metazoans. Mitotic spindle assembly in vertebrates involves anti-parallel MT-MT interaction in addition to chromatin/kinetochore-MT interaction 50. Such parallel pathways often employ similar MAPs and motor proteins that promote and stabilize the MT assembly to drive the formation of a fusiform spindle array 51. Dismantling one, e.g. the centrosome-based pathway perhaps would not jeopardize the birth of a bipolar mitotic spindle that could be assembled based on chromosome/kinetochore-based MT nucleation.

It has been learned in plant cells that lagging chromosome caused by errors in anaphase can induce MT nucleation/assembly in to discrete kinetochore bers described as minispindles 52. This minispindle phenomenon also can be observed in grasshopper spermatocyte meiosis 53. Such an isolated kinetochore fiber pair or a minispindle, resembling the microtubular fir tree (MTFT) in Haemanthus cells, are self-autonomous and join together to form “a forest of MTFTs” resembling the acentrosomal spindle apparatus 54. This unifying effect was thought to be self-sufficient by MTFTs themselves although the underlying mechanism has not been demonstrated 46. Our results support the notion that MTs organized by one or more motors like Kinesin-14D1 contribute to the organization of minispindles into a mitotic spindle with chromosomes attached and aligned. Here, our results demonstrated that inside a plant mitotic spindle, the Kinesin-14D1 motor specifically acted on and cross-linked anti-parallel MTs in the spindle midzone throughout mitosis and served as the first specific marker for the midzone which likely represented the interdigitation zone as early as the end of prophase and the beginning of prometaphase. The MTFT-derived bulk of spindle MTs, because of being bipolar, were sufficient for sister chromatid segregation, despite lacking the organized convergence in the kinesin-14d1 mutant cells (Figure 9D). The bipolar organization of these MTFTs or minispindles were dependent on highly dynamic MTs as demonstrated by the vulnerability to low doses of oryzalin (Figure 9E). This is consistent with the phenomenon observed in the endosperm spindle as reported 49.

Our findings also echoed an earlier hypothesis that spindle assembly involves motor-driven organization of MTs at or near the chromosomes as well as in the peripheral region surrounding the chromosome mass 55. Although chromosome-dependent MT organization often is emphasized when spindles are drawn, chromosome-independent spindle MTs perhaps constitute the other equally important part of the spindle apparatus. In centrosome-bearing animal cells, these MTs may have been initiated from the MTOC structure and persisted through anaphase, perhaps as the counterpart of the prophase spindle in
plant cells. It has been revealed that centrosomes and chromosomes contribute in parallel to spindle assembly and remodeling at M phase \(^{53}\). While either one is dispensable, the surgical removal of both leads to a complete collapse. This was further manifested by the observed “anaphase” and cytokinesis when chromosomes were taken out of the grasshopper meiotic cell by micromanipulation \(^{56}\). When the chromosomes were removed, kinetochore fibers no longer persisted. Although it was not tested, it was likely that chromosome independent MTs originated from two poles/centrosomes underwent the remodel process towards cytokinesis. Obviously, MT motors that are not associated with chromosomes/kinetochores, like Kinesin-14D1 reported here, would be responsible for the reorganization process.

Our results also brought new insights into the organization of the spindle midzone that is often said to be dependent on MAPs and/or kinesins acting specifically on interdigitated, anti-parallel MTs \(^{57}\). The central player perhaps is the MT crosslinker belonging to the MAP65/PRC1/Ase1 family, discovered first in tobacco cells \(^{58}\). In \(A.\) \textit{thaliana}, there are nine functionally diversified MAP65 isoforms among which MAP65-3 and MAP65-4 are expressed in cell cycle-dependent manners and act in the spindle midzone by cross-linking anti-parallel MTs \(^{41, 59}\). A revised model recently suggests that the spindle midzone is organized by so-called “bridging MTs” branched out from the kinetochore fibers that are cross-linked by PRC1 in vertebrate cells, instead of having the conventional “interpolar” or anti-parallel MTs initiated from two poles \(^{60}\). Here we showed that Kinesin-14D1 cross-linked anti-parallel MTs that are separated from those cross-linked by MAP65-4. Furthermore, although MAP65-3 is required for the localization of factors like Kinesin-12 to the spindle/phragmoplast midzone \(^{61}\), Kinesin-14D1 was still detected there in the \textit{map65-3} mutant cells. In contrast to vertebrate cells in which the PRC1 accumulation towards MT plus ends is thought to be coupled with the function of the Kinesin-4 motor \(^{62}\), plant cells seem to be able to construct functional spindle apparatus without Kinesin-4 as the loss of all three Kinesin-4 genes did not prevent vegetative growth and reproduction in \(A.\) \textit{thaliana} \(^{63}\). Instead, the function of Kinesin-4 seems to be reserved for shortening the overlapping zone of anti-parallel MTs in the phragmoplast in moss cells \(^{64}\). Taken together, we concluded that both the classical anti-parallel MTs and the bridging MTs are present in the plant spindles, and they are cross-linked by different factors so that they contributed to MT stabilization/organization in the spindle midzone independently from each other in plant mitosis.

**Functional specialization among the three Kinesin-14D motors**

The sequence divergence of the Kinesin-14 motors in six classes, as reflected by the distinct functional domains, leaves little if any doubt that they must interact with different partners for their different intracellular motile activities. Within a single class, however, different isoforms more likely exercise a unified function. However, our live-cell imaging data together with functional analysis suggested that the Kinesin-14D motors have their functions further differentiated although being associated with spindle MTs. While acquiring different localization patterns prior to the anaphase onset, they all decorated MT
bundles in the spindle midzone. Unlike Kinesin-14D1 which excluded kinetochore fibers, both Kinesin-14D2 and Kinesin-14D3 decorated kinetochores. This dynamic feature was mostly described for proteins in the chromosomal passenger complex (CPC) which regulates chromosome congression during mitosis and actomyosin contractile ring during cytokinesis in animal cells. The CPC homologs have been detected at the mitotic centromeres and the midzones of anaphase spindles and phragmoplasts in Arabidopsis thaliana for an essential function in mitosis. In animal cells, the spindle midzone localization of the CPC is dependent on the Kinesin-6 motor. It would be interesting to test whether the Kinesin-14D motors may be functionally coupled with the CPC in Arabidopsis thaliana.

How do the three Kinesin-14D motors acquire intriguingly different dynamic localizations during mitosis? We were surprised to learn that Kinesin-14D1 and Kinesin-14D2 acted differently although carrying similar domain structures besides the high homology in the motor domain. It will be particularly interesting to learn what determines the kinetochore localization of Kinesin-14D2 and Kinesin-14D3. In animal cells, cytoplasmic dynein but not Kinesin-14, as MT minus end-directed motors, is detected at the kinetochores. Currently, it has been advocated that the great expansion of the Kinesin-14 motors in flowering plants is attributed to the loss of cytoplasmic dynein. Alternatively, we hypothesize that early eukaryotes, similar to algae like Chlamydomonas reinhardtii, perhaps already had kinetochore-associated Kinesin-14D, instead of cytoplasmic dynein. In animals, however, the loss of most Kinesin-14 motors might have led to the re-designation of cytoplasmic dynein for a kinetochore function through the employment of kinetochore specific adaptor proteins. To elucidate the basis of the different localizations of these three closely related motors, on the other hand, it would be necessary for us to dissect whether the domains in Kinesin-14D2 corresponding to those in Kinesin-14D1 tested here serve as localization determinants during mitosis. Motor adaptors serve the roles of not only subcellular targeting but also activation. Therefore, identification of such adaptor proteins would advance our knowledge of the mitotic functions of these novel motors.

Taken together, the three Kinesin-14D motors likely play different roles in mitosis and perhaps in cytokinesis because of their distinct dynamic behaviors on clearly different MT populations in Arabidopsis thaliana. While Kinesin-14D1 was associated with kinetochore fiber independent MTs and functioned in engaging individual kinetochore fiber complexes, the other two Kinesin-14D motors, despite the homologies in their primary amino acid sequences, perhaps functioned differently at kinetochores and/or kinetochore fibers. It would also be interesting to learn whether these Kinesin-14 motors have any redundant functions with motors in other classes of Kinesin-14.

Methods

Phylogeny Reconstruction of Kinesin14 motors

Amino acid sequences of Kinesin-14 motors were retrieved from the GenBank (accession numbers are included in Supplemental Table 1). Their conserved motor domains were identified using Multiple Expectation maximizations for Motif Elicitation (MEME) tool and trimmed prior to being aligned by using
the ClustalX multiple sequence alignment tool. The outcomes were used to generate a phylogenetic tree by using neighbor-joining method in MEGA5. All branches are statistically supported through 1000 bootstrap tests.

**Plant Materials, Growth Conditions, Transient Expression and Transformation**

T-DNA insertional lines of *A. thaliana* were acquired from the Arabidopsis Biological Research Center (ABRC) located at Ohio State University in Columbus, Ohio. They are *kinesin-14d1* (SALK_064973C at the At2g22610 locus), *kinesin-14d2* (SALK_072497C at At1g72250), and *kinesin-14d3* (SALK_202625C at At5g27550). The primers used for genotyping the mutant plants are: 64973LP (5’- TTA GAG TTT CAC TCA CAT CAT GCT C -3’) and 64973RP (5’- TGA AGT TGC TAG AGA AAG GAG AGA G -3’) for SALK_064973C, 72497LP (5’- ATA GTT TGC TCT TTG TGC ATT CAC -3’) and 72497RP (5’- CAG AAT TGC ATG AAA ATC TCA AAG -3’) for SALK_072497C, and 202625LP (5’- AAA CCT CCA ACA TGC TAA CTG ATA G -3’) and 202625RP (5’- TTA CAA GTG GAA ACC AAG AAG TTT C -3’) for SALK_202625C. The T-DNA specific primer is LBb1.3: 5’- ATT TTG CCG ATT TCG GAA C-3’. The map65-3 mutant line was also described as published. The *A. thaliana* plants were grown on soils in chambers with a cycle of 16-hr light and 8-hr dark at 70% relative humidity at 22°C. Seedlings for live-cell imaging and immunolocalization experiments were produced on solid medium supplied with ½ Murashige Skoog (MS) salt mixture and 0.8% Phytagel (Sigma).

In the oryzalin sensitivity test, seeds were sown on ½ MS salt mixture and 0.8% Phytagel with the addition of 100 nM oryzalin in DMSO while equal volumes of DMSO served as the negative control. Plants are subjected to cold stratification for 2 days prior to being moved to chambers and to grow for 21 days. For short term oryzalin treatment of seedlings, plants were germinated on the ½ MS salt mixture and 0.8% Phytagel for 5-6 days, then transferred to solid media containing either 100 nM oryzalin or DMSO for 2 hours before live-cell imaging or immunofluorescence.

Tobacco (*N. bethamiana*) plants were grown in a growth chamber with a cycle of 16-hr light and 8-hr dark at 25°C. The CDELS has leaf cells induced to enter mitosis by cyclin D expression as described previously. The *Agrobacterium tumefaciens* strain GV3101 was used for both leaf infiltration experiments in tobacco and floral dipping-assisted transformation in *A. thaliana*. Tobacco epidermal cells excised leaf segments were observed 48 hrs after infiltration and *A. thaliana* root cells were observed 4 days after germination.

**Construction of Expression Vectors**

To produce a Kinesin-14D1-GFP construct, a 5589-bp genomic fragment, containing a 503-bp hypothesized promoter region, was amplified using the primers II22610F (5’-CAC CAT GAT TGC GTT TTG
CAA CTA CTT TTG GTT G-3') and II22610R (5'-TGC GCC TGC GCC TCT TGA CCA AAT CTT CTT GGA GTC TC-3'). The fragment was inserted into pENTR-D/TOPO vector (Thermo Fisher) according to the manufacturer's instruction. The resulting pENTR-II22610 plasmid was used for an LR recombination reaction with the pGWB4 plasmid \(^71\), resulting in the plasmid pGWB4-II22610 in which GFP-coding sequence was fused in-frame with the last exon of \textit{Kinesin-14D1} after removal of the stop codon.

Both Kinesin-14D2-GFP and Kinesin-14D3-GFP expression constructs were made using a modified entry vector of pCH1 containing the GFP-coding sequence preceded by a 6X GSS (glycine-serine-serine) linker-coding sequence, amplified from the pUC35S-ACT7-GSSX6-GFP plasmid \(^72\), using primers of GSS6-sGFP5 (5'-GTC TGC AGG TCG ACG AAT TCG CGG CCG CAC TCG AGG TTC TTC TGG TTC ATC TGG ATC CTC-3') and GSS6-sGFP3 (5'- CTT TGT ACA AGA AAG CTG GGT CTA GAT ATC TTA CTT GTA CAG CTC GTC CAT GCC GT-3') and inserted into the XhoI site in pENTR4 (Thermo Fisher). The 7258-bp genomic fragment containing 2336-bp promoter region of \textit{Kinesin-14D2} was amplified by using primers of I72250F (5'-CAA TTC AGT CGA CTG GAT CCG GTA CCC TTG GTA CGT GTT ACA TCA AG-3') and I72250R (5'-AGA GGA TCC AGA TGA ACC AGA AGA ACC GAT CCA TCG CTC TTG TTT CTG CG-3'). Similarly, a 4121-bp full-length genomic fragment, containing 434-bp of the promoter region, of \textit{Kinesin-14D3} was amplified using V27550F (5'- GGA ACC AAT TCA GTC GAC TGG ATC CGG TAC GAG GCA AGC CTT TGG CAA ATC-3') and V27550R (5'- TCC AGA GGA TCC AGA TGA ACC AGA AGA ACC TTG TTC CCT CCG AAG CAA GTC AG-3'). These fragments were inserted into pCH1 linearized by XhoI and KpnI by Gibson assembly (New England Biolabs). The resulting entry clones were recombined with pGWB1 by LR clonase (Thermo Fisher) to give rise to the final Kinesin-14D2-GFP and Kinesin-14D3-GFP expression vectors.

All truncated proteins of Kinesin-14D1 were expressed under the native \textit{Kinesin-14D1} promoter by using the pENTR-II22610 plasmid described above as the template. The pENTR-II22610\(^\Delta\text{motor}\) vector was produced by using primers II22610DmotorF (5'-ACC GGT GAG ATC CAG AAG TTG-3') II22610DmotorN (5'-TGC TTG CTC TTC ACT GTA CTT CTG-3') and the fragment was circularized by blunt end ligation using KLD enzyme mix (New England Biolabs). The primer pairs of NvecrF (5'-GGC GCA GGC GCA AAG GGT GG-3') and II22610DmotorN were used to linearized fragment of a plasmid with the N terminal region of Kinesin-14D1. This fragment is circularized by blunt end ligation to give rise to pENTR-II22610\(^N\). Similarly, the primers of II22610DmotorF and II22610CR (5'-CAT AAT TAT TTG GAA GCA AAT CGT CTC TC-3') were used to generate pENTR-II22610\(^C\). The pENTR-II22610\(^{C1}\) plasmid was produced by amplifying the C-terminal genomic fragment excluding the coil-coil domain, using the primers II22610CF (5'-CAG AGA GAC GAT TTG CTT CCA AAT AAT TAT GCC TGA AGT TGC AAA CGC TAC G-3') and II22610R. The plasmid backbone was amplified using the primers Nvecr and II22610CR. This backbone and the amplified C-terminal fragment were combined by Gibson assembly to generate pENTR-II22610\(^{C1}\). These pENTR vectors are shuttled into pGWB4-based vector by LR clonase, to yield a translational fusion with a C-terminus GFP.

To construct the MAP65-4-mCherry binary vector, the pENTR-MAP65-4 plasmid \(^41\) was used for an LR clonase-catalyzed recombination with pCH5 containing the mCherry-coding sequence. The binary
vectors of histone H1-TagRFP, mCherry-TUB6, and GFP-HTR12/CENH3 were described as published 40,73.

**Immunofluorescence and Confocal Microscopy**

Germinated seedlings were fixed and processed for immunolocalization experiments as described previously 74. Antibodies include the DM1A anti-α-tubulin monoclonal antibody (Millipore Sigma) and affinity purified polyclonal anti-GFP antibodies (Kong et al., 2010). These primary antibodies were detected by fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG and TexasRed-conjugated donkey anti-mouse IgG antibodies, respectively, while DNA was labeled by the fluorescent dye DAPI at 1 µg/ml. Image were acquired with a Plan-Fluar 100x objective of an Eclipse 600 epifluorescence microscope (Nikon).

Live-cell imaging was performed on an LSM710 laser scanning confocal module mounted on an Axio Observer inverted microscope by using a 40X water immersion objective (Carl Zeiss). Samples of tobacco leaves and *A. thaliana* roots were prepared and observed as described previously 40. Acquired images were pseudo-colored and merged in Metamorph software (Thermo Fisher) and assembled in Adobe Photoshop (Adobe) for the final presentation.

**Declarations**

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**Figures**
Figure 1

Six classes of Kinesin-14 motors in representative organisms. (A) Illustration of the appearance of classes A-F of Kinesin-14 motors in the selected organisms. (B) Summary of the presence or absence of the six classes Kinesin-14 motors in the chosen organisms. Classes A and B are detected in G. lamblia while the simple green alga C. reinhardtii possesses classes A-D. Class E Kinesin-14 first appeared in the green alga K. nitens and class F in the moss P. patens. All six classes are present in higher plants. In
contrast, only class A is present in fungi represented by A. nidulans, and vertebrates represented by the mouse M. musculus have classes A and D. (C) Domain structure of three Kinesin-14D motors in A. thaliana.

Figure 2

Cell cycle-dependent dynamics of Kinesin-14D1 (K14D1) revealed by the tobacco CDELS mitotic cells. (A) Kinesin-14D1-GFP (green) localization with chromatin (magenta) marked by a histone H1-TagRFP fusion
protein. At prophase, Kinesin-14D1 is exclusively present in the nucleoplasm with no obvious association with the chromatin. The motor starts to accumulate in spaces between condensed chromosomes in the central region of the spindle apparatus at prometaphase right after nuclear envelope breakdown. When chromosomes are aligned at metaphase plate, Kinesin-14D1 remains in the midzone in a coalesced pattern intercalated between the chromosomes. At anaphase when sister chromatids arrive at the two spindle poles, Kinesin-14D1 remains in the central zone. The motor becomes highly restricted in a narrow region when daughter nuclei reform during cytokinesis. (B) Dual localizations of Kinesin-14D1 (green) and MTs (magenta, marked by mCherry-TUB6) at different stages of mitotic division. Kinesin-14D1 remains in the nucleus when the cell establishes the preprophase band. When a bipolar prophase spindle emerges, the motor is enriched in the midzone and avoids more abundant MTs towards two poles. When the mitotic spindle is developed, the Kinesin-14D1-GFP signal coalesces and is restricted to MTs in the spindle midzone. At anaphase when kinetochore fibers shorten, Kinesin-14D1 remains in the spindle midzone when new MTs are made. In the cell undergoing cytokinesis, Kinesin-14D1 exclusively localizes to the midzone of the phragmoplast. (C) Enlarged view of Kinesin-14D1 localization in the spindle midzone in a cell at early anaphase. Images on the left have the entire spindle region included and the boxed region is enlarged in images on the right. The Kinesin-14D1 signal often juxtaposed to the prominent MT bundles, as revealed in the fluorescence intensity scan. The relative fluorescence intensity values are arbitrarily normalized. Scale bars, 5 µm (A and B) and 2 µm (C).
Figure 3

Cell cycle-dependent dynamics of Kinesin-14D2 (K14D2) revealed by the tobacco CDELS mitotic cells. (A) Kinesin-14D2-GFP (green) localization with chromatin (magenta) marked by a histone H1-TagRFP fusion protein. At prophase, Kinesin-14D2 is diffuse in the nucleoplasm. Around the time of nuclear envelope breakdown, the fluorescent signal of Kinesin-14D2 motor appears in a prophase spindle configuration. When chromosomes are aligned at metaphase plate, Kinesin-14D2 accumulates on elaborated fibers and
is pronounced at kinetochore-like points. At anaphase when sister chromatids arrive at the two spindle poles, Kinesin-14D2 is most noticeable in the spindle midzone. Later, the motor becomes highly restricted in a narrow region when daughter nuclei reform during cytokinesis. (B) Dual localizations of Kinesin-14D2 (green) and MTs (magenta, marked by mCherry-TUB6) at different stages of mitotic division. Kinesin-14D2 remains in the nucleus and does not overlap with preprophase band or perinuclear MTs. When a bipolar prophase spindle emerges, the motor is distributed across the spindle MTs and is more marked towards two poles. At metaphase when the mitotic spindle is established, Kinesin-14D2-GFP colocalizes with kinetochore fibers towards the metaphase plate and kinetochores. At anaphase with kinetochore fibers shortened, Kinesin-14D2 is restricted in the spindle midzone. At cytokinesis, finally, Kinesin-14D2 exclusively localizes to the midzone of the phragmoplast. (C) Enlarged views of two metaphase cells in (A) and (B) that highlight Kinesin-14D's association with the kinetochores on the chromosomes (top) and at the end of kinetochore fibers (bottom). Scale bars, 5 µm (A and B) and 2 µm (C).
Cell cycle-dependent dynamics of Kinesin-14D3 (K14D3) revealed by the tobacco CDELS mitotic cells. (A) Kinesin-14D3-GFP (green) localization with the reference of chromatin (magenta) marked by a histone H1-TagRFP fusion protein. At prophase, Kinesin-14D3 is enriched on the nuclear envelope. At prometaphase, the Kinesin-14D3-GFP fusion are enriched in MT-like signals surrounding chromosomes. When chromosomes are aligned at metaphase plate, Kinesin-14D3 is most noticeable on kinetochore...
fibers with signals gradually diminished away from the middle of the cell. After sister chromatids are separated, Kinesin-14D3 is most pronounced in the central zone while still being detected at the kinetochores. Towards the end of anaphase, the motor is prominently detected in the spindle midzone but not on kinetochores anymore. During cytokinesis, Kinesin-14D3 becomes highly restricted in a narrow region when daughter nuclei reform. (B) Dual localizations of Kinesin-14D3 (green) and MTs (magenta, marked by mCherry-TUB6) at different stages of mitotic division. Kinesin-14D3 colocalizes with the perinuclear MTs at prophase. At prometaphase, the motor is detected in the central region where MTs from two poles meet. At metaphase, the Kinesin-14D3-GFP signal is mostly detected on kinetochores and faint signals briefly trailing along kinetochore fibers. At anaphase when kinetochore fibers shorten, Kinesin-14D3 is mostly enriched in the spindle midzone where new MTs are developed and decorates segregated kinetochores. Towards the end of anaphase as marked with kinetochore fibers nearly undetectable, Kinesin-14D3 is exclusively associated with the spindle midzone. Finally, Kinesin-14D3 appears in the midzone of the phragmoplast. (C) Enlarged views of two anaphase cells in (A) and (B), highlighting Kinesin-14D3 association with the kinetochores on the chromatids (top) and with both MT bundles in the middle and segregated kinetochores (bottom). Scale bars, 5 µm (A and B) and 2 µm (C).
Figure 5

Kinesin-14D1 is required for the birth of mitotic spindles with unified, converged poles in A. thaliana. (A, B) The loss of Kinesin-14D1 causes hypersensitivity to 100 nM oryzalin. Without MT depolymerization challenges, the kinesin-14d1 (k14d1) mutant seedlings grow indistinguishable from the wild-type control (WT) as well as the mutant expressing the Kinesin-14D1-GFP fusion protein (k14d1 + K14D1). The kinesin-14d1 mutant exhibits lethality and fail to have roots extended in the presence of 100 nM oryzalin.
while both the control and rescued seedlings show relatively normal growth. (C) Snapshots of mitotic MT arrays in a kinesin-14d1 (k14d1) mutant cell by live-cell imaging. Note that the cell formed preprophase band and a relatively normal prophase spindle, but its metaphase and anaphase spindles have wide spindle poles with little if any sign of convergence. The spindle MT array is eventually replaced by a phragmoplast array. The time stamps (hr:min:sec) have the point of nuclear envelope breakdown set at 00:00:00. Spindle MT arrays are restored with converged poles upon the expression of Kinesin-14D1-GFP in the mutant (k14d1 + K14D1). (D) Assessment of the pole width in metaphase spindles by anti-tubulin immunofluorescence in the kinesin-14d1 (k14d1) mutant, mutant expressing Kinesin-14D1-GFP (k14d1 + K14D1), and the wild-type control. Spindle pole width is assessed as the ratio of pole width/spindle width at the equatorial plane. The mutant produces spindles with pole width significantly wider than those of the rescued or wild-type control. (E) Live-cell imaging of mitotic MT arrays in the kinesin-14d1 (k14d1) mutant and the mutant expressing Kinesin-14D1-GFP (k14d1 + K14D1). The time stamps (hr:min:sec) are set according to the point of nuclear envelope breakdown as 00:00:00. While the cell of the rescued plant survived the oryzalin treatment and enters into anaphase at around 00:23:11, the mutant cell has MT bundles randomly placed even after 00:31:05. (F) Examined by anti-tubulin immunofluorescence, the kinesin-14d1 mutant has spindles collapsed in the presence of 100 nM oryzalin while the rescued line and wild-type control produce relatively normal spindles under identical conditions, as examined. Quantitative assessment shows over 50% of the kinesin-14d1 spindles collapse when treated with oryzalin while both the rescued plants and the wild-type control had significantly fewer cells exhibiting abnormal spindles. Scale bars, 1 cm (A and B) and 5 µm (C-F).
Figure 6

Kinesin-14D1 possesses functional distinct domains as tested in the tobacco CDELS cells. (A) Diagrammatic representation of the Kinesin-14D1 domains as in Figure 1C. Different truncations are constructed by including one or more segments of the motor, in GFP fusions under the control of the original promoter when co-expressed with mCherry-TUB6 to mark MTs. (B) The truncation without the motor domain (K14D1ΔM) resides in the nucleus and decorates both spindle and phragmoplast MTs.
(C) The N-terminal polypeptide including both the malectin domain and two coiled-coils (K14D1N) shows diffuse nuclear localization and evenly presence in both the spindle and phragmoplast regions. (D) The polypeptide C-terminus to the motor domain (K14D1C) appears in the nucleus and decorates both chromosomes and spindle MTs at metaphase and phragmoplast MTs and daughter nuclei at cytokinesis. (E) The smaller C-terminal domain excluding the coiled-coil (K14D1C') is detected inside the prophase nucleus, metaphase chromosomes and daughter nuclei. Scale bars, 5 µm.
Kinesin-14D1 selects a distinct set of midzone MTs inside the mitotic spindle as revealed in the tobacco CDELS cells. (A) When a Kinesin-14D1-mCherry fusion protein (K14D1) is co-expressed with a GFP-HTR12 fusion protein (CENH3) marking the centromeres that often do not overlap with the motor-highlighted MT bundles at an earlier stage of mitosis (top) and around metaphase when the centromere pairs are aligned at the metaphase plate. Note that Kinesin-14D1-highlighted signals towards the periphery often do not have the centromere signal overlapped. (B) When MAP65-4-mCherry is co-expressed with GFP-HTP12, the highlighted centromeres typically sit on MAP65-4-labeled MT bundles at both prometaphase (top) when they have not been organized and metaphase when the centromere pairs are aligned at the equatorial plane (bottom). (C) Comparative localizations of Kinesin-14D1-GFP and MAP65-4-mCherry in the spindle midzone. The two signals often differ in terms of the highlighted length of MT bundles and selection of MT bundles across the spindle midzone. Such differences become more pronounced when the images are binarized (bottom). Fluorescence intensity scan further reveal the difference of two signals, especially in the regions highlighted by asterisks (*). (D) MAP65-4 decorates major MT bundles in the spindle midzone. MAP65-4-GFP is detected along the major MT bundles in the spindle midzone at anaphase, which is clearly demonstrated by fluorescence intensity scans. Scale bars, 5 µm.
Figure 8

The Kinesin-14D1 function in the assembly of the mitotic spindle MT array in plant cells. (A) A bipolar prophase spindle is established before nuclear envelope breakdown, in part by the action of Kinesin-14D1 in the midzone. (B) After nuclear envelope breakdown, MTs are nucleated from the chromosomes/kinetochores and/or from the cytoplasm that are captured by the kinetochores in order to establish the minispindle or MTFT on each chromosome. (C) The integration of chromosome-based MT assembly and the MT array inherited from the prophase spindle gives rise to the mitotic spindle MT array. (D) When Kinesin-14D1 is lost, minispindles or MTFTs become disorganized and dissociate from each other. (E) The treatment of the kinesin-14d1 mutant with 100 nM oryzalin breaks down the connection among minispindles or MTFTs so that the spindle MT array collapses.
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