RNAi Screening Identifies the Armadillo Repeat-Containing Kinesins Responsible for Microtubule-Dependent Nuclear Positioning in Physcomitrella patens

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Proper positioning of the nucleus is critical for the functioning of various cells. Actin and myosin have been shown to be crucial for the localization of the nucleus in plant cells, whereas microtubule (MT)-based mechanisms are commonly utilized in animal and fungal cells. In this study, we combined live cell microscopy with RNA interference (RNAi) screening or drug treatment and showed that MTs and a plant-specific motor protein, armadillo repeat-containing kinesin (kinesin-ARK), are required for nuclear positioning in the moss Physcomitrella patens. In tip-growing protonegmal apical cells, the nucleus was translocated to the center of the cell after cell division in an MT-dependent manner. When kinesin-ARKs were knocked down using RNAi, the initial movement of the nucleus towards the center took place normally; however, before reaching the center, the nucleus was moved back to the basal edge of the cell. In intact (control) cells, MT bundles that are associated with kinesin-ARKs were frequently observed around the moving nucleus. In contrast, such MT bundles were not identified after kinesin-ARK down-regulation. An in vitro MT gliding assay showed that kinesin-ARK is a plus-end-directed motor protein. These results indicate that MTs and the MT-based motor drive nuclear migration in the moss cells, thus showing a conservation of the mechanism underlying nuclear localization among plant, animal and fungal cells.

Keywords: Kinesin • Microtubule • Nuclear positioning • Physcomitrella patens • RNAi screening.

Abbreviations: DMSO, dimethylsulfoxide; EMCCD, electron-multiplying charge-coupled device; GFP, green fluorescent protein; kinesin-ARK; Armadillo repeat-containing kinesin; dsRNA, double-stranded RNA; mRFP, monomeric red fluorescent protein; MT, microtubule; NE, nuclear envelope; qRT–PCR, quantitative real-time reverse transcription–PCR; RFP, red fluorescent protein; RNAi, RNA interference; TIRFM, total internal reflection fluorescence microscope.

Introduction

Eukaryotic cells have a nucleus that envelops genetic material along with regulators of gene expression. In many cell types, the nucleus is not randomly positioned in a cell (Gundersen and Worman 2013). For example, cells of rod-shaped fission yeast keep the nucleus at the center of the cell, and consequently form the mitotic spindle and the cytokinetic contractile ring at the cell center; these mechanisms ensure symmetrical cell division (Tran et al. 2001). In contrast, in many types of differentiated animal cells such as epithelial cells and neurons, the nucleus is positioned asymmetrically, which might be necessary for the cell’s physiology (Gundersen and Worman 2013). Such altered positioning of the nucleus is also observed in plant cells, sometimes brought about in a regulated manner. For example, blue light-dependent nuclear positioning has been shown in Arabidopsis leaf cells (Iwabuchi et al. 2007, Iwabuchi et al. 2010).

Previous studies have revealed that microtubules (MTs) that are connected to the nuclear envelope (NE) are key cytoskeletal players in many animal and yeast cells. Nuclear-attached MTs can move the nucleus via two mechanisms. First, the plus end of MTs interacts with the cell cortex and generates either a pushing or a pulling force. In fission yeast, centering of the nucleus is achieved through pushing of cell tips by MTs (Tran et al. 2001), whereas cells of budding yeast translocate the nucleus to the bud neck through MT pulling at the cell cortex, near which the minus-end-directed motor called cytoplasmic dynein is concentrated (Adames and Cooper 2000). The second commonly observed mechanism of MT-driven nuclear positioning is dependent on the motor proteins that connect the NE and MTs. For example, female pronuclear movement is caused by nucleus-associated dynein that walks along the MTs coming out of the sperm centrosome (Reinsch and Gonczy 1998). Processive plus-end-directed motors kinesin-1 and kinesin-3 have also been shown to be responsible for nuclear movement; specific proteins at the NE surface recruit these motor proteins (Fridolfsson and Starr 2010, Tsai et al. 2010). In plants, on the other hand, actin and the associated myosin motor protein (e.g. myosin XI-i) control nuclear positioning in many cell types (Chytilova et al. 2000, Iwabuchi et al. 2010, Tamura et al. 2013). Nevertheless, the adaptor proteins on the NE are conserved among yeast, animal and plant cells; the SUN-KASH protein complex that is embedded in the NE is associated with plant myosin or animal kinesin/dynein (Gundersen and Worman 2013, Tamura et al. 2013). To our
knowledge, kinesin-dependent nuclear translocation has not been identified in land plant cells (note that land plants do not possess cytoplasmic dynein).

The protonemal cell of the moss Physcomitrella patens is an emerging model system for plant cell biology, e.g. for analysis of the molecular mechanism underlying cell growth and mitosis (Cove 2005, Vidali et al. 2007, Hiwatashi et al. 2008, Vidali et al. 2009, Nakaoka et al. 2012, Kosetsu et al. 2013, Miki et al. 2014). The rate of homologous recombination is very high, so that gene disruption and green fluorescent protein (GFP, or its variant) tagging of the endogenous gene are easy to perform; this approach quickly provides insight into gene functions. As an additional tool for functional studies, we recently developed a conditional RNA interference (RNAi) system, in which a knockdown of a single or multiple homologous genes can be induced in protonemal cells; we used this system to evaluate the effects of down-regulation of genes essential for mitosis (Nakaoka et al. 2012, Kosetsu et al. 2013, Miki et al. 2014). Another key feature of the P. patens protonemal system is the ease of live cell microscopy. The cells are found in a single layer and actively grow and divide every 5–6 h; thus, they can be studied using high-resolution confocal microscopy and prolonged imaging (>12 h) (Cove 2005, Prigge and Bezanilla 2010, Nakaoka et al. 2012, Miki et al. 2014). Combining microscopy and loss-of-function analysis in protonemal cells has provided new insights into gene functions and the molecular mechanisms of certain cellular processes (Vidali et al. 2007, Augustine et al. 2008, Hiwatashi et al. 2008, Nakaoka et al. 2012, Kosetsu et al. 2013, Miki et al. 2014). Recently, the genome of P. patens was analyzed, and 78 kinesin superfamily genes were identified (Shen et al. 2012, Miki et al. 2014). Based on the amino acid sequences, P. patens kinesin genes were classified into 10 subfamilies. Physcomitrella patens does not possess kinesin-1 or kinesin-3 subfamily genes, which in animal and fungal cells play a major role in cargo transport, e.g. the nucleus (Hirokawa et al. 2009, Zekert and Fischer 2009). We attached a Citrine tag (a GFP variant) to the C-terminus of virtually all kinesins, performed time-lapse microscopy, and identified 43 kinesins associated with the mitotic apparatus such as spindles, phragmoplasts and chromosomes (Miki et al. 2014). Because most of the kinesins show inconsistent localization patterns compared with the animal homologs, the function of each kinesin in P. patens is not yet clear. Moreover, the study suggested that many kinesins, including the mitotic kinesins, are also expressed in interphase (Miki et al. 2014); however, the function of these motors in interphase is also unknown.

In this study, we first showed that MTs, but not actin filaments, are required for nuclear centering after cell division in the protonemal cells of P. patens. We then performed comprehensive RNAi screening that targeted all 78 kinesins and found that depletion of armadillo repeat-containing kinesins (kinesin-ARKs) affects nuclear positioning. In the absence of kinesin-ARKs, the daughter nucleus in apical cells fails to reach the cell center and is instead mislocalized to the cell plate-proximal region, concomitantly with the loss of MT bundles around the nucleus. The kinesin-ARK protein is localized to the cytoplasmic MTs in vivo and shows MT gliding activity in vitro. Taken together, these results suggest that MTs and kinesin-ARK are crucial for nuclear migration.

Results

The nucleus is translocated to the center in an MT-dependent manner after cell division

In a study by Doonan and colleagues, protonemal apical cells were treated for ≥1 h with an MT-destabilizing drug, cremarid, and the crucial function of MT in tip growth was demonstrated based on the terminal phenotype (Doonan et al. 1988). We repeated this experiment using oryzalin, a compound that has seen frequent usage in recent times (Finka et al. 2007, Hiwatashi et al. 2014), cells expressing GFP–tubulin and histone H2B–monomeric red fluorescent protein (mRFP) (Nakaoka et al. 2012), and time-lapse imaging along with drug addition. We confirmed the previous finding of the tip growth defect (Finka et al. 2007, Hiwatashi et al. 2014) and observed, as expected, the absence of spindle formation during mitosis (Supplementary Movie S1). Histone–RFP imaging indicated that the majority of the cells in interphase retain their nuclei in the center of the cell, while the tip growth was suppressed (Supplementary Movie S1). During the course of this experiment, we found that nuclear movement just after nuclear division is affected by oryzalin treatment. In control dimethylsulfoxide (DMSO)-treated cells, daughter nuclei were translocated towards the cell center immediately after cell division (Fig. 1A, D; Supplementary Fig. S1; Supplementary Movie S2). The nucleus was then kept in the middle of the cell because it was moved towards the cell tip at the rate of tip growth (Supplementary Fig. S1). In contrast, when oryzalin was added to telophase cells, daughter nuclei were not properly translocated, but rather they stayed at the same position (Fig. 1B, D; Supplementary Movie S2). This phenomenon was not an indirect effect of the drug on tip growth because treatment with actin inhibitors such as latrunculin B or cytochalasin B (Sato et al. 2001, Harries et al. 2005, Finka et al. 2007, Vidali et al. 2009, Wu and Bezanilla 2014) also suppressed tip growth with no significant effect on nuclear translocation (Fig. 1C, D; Supplementary Movie S2). These results indicated that nuclear translocation after cell division was dependent, at least partially, on MTs in the apical cell.

RNAi screening identified kinesin-ARKs as the motor proteins required for proper nuclear positioning

The oryzalin experiment suggested that there were at least three activities in the protonemal apical cell that required MTs: mitosis, tip growth and nuclear migration. Because these activities may involve generation of force by motor proteins, we designed an RNAi screening method to identify kinesin superfamily proteins involved in each process (Fig. 2; Supplementary Movie S3).

Physcomitrella patens has 78 kinesins, 69 of which are classified into 10 subfamilies and the remaining nine are unclassified
We designed two RNAi constructs for each gene. We amplified approximately 700 bp fragments from the cDNA pool, and cloned them into the pGG626 vector, which is an integration vector for inducible RNAi (Nakaoka et al. 2012). RNAi-pGG626 constructs were introduced into the GFP–tubulin/histone–RFP cell line, and stable clones were selected. Based on histone–RFP intensity, which is an indicator of RNAi penetrance, we selected about five candidate RNAi lines corresponding to each construct [RFP intensity is expected to be reduced concomitantly with a knockdown of the target protein (Nakaoka et al. 2012)]. In total, we selected >450 RNAi lines, in which 61 genes were targeted. We could not select RNAi lines for the remaining 17 genes mostly because we were unable to amplify these gene fragments. Expression of double-stranded RNAs (dsRNAs) was induced by turning on the promoter using β-estradiol treatment. On days 5–6, time-lapse imaging was performed for >6 h (3 min intervals), followed by manual examination of the phenotype by watching the cell videos. During analysis of >3,000 movies, we detected defects in mitosis as a result of a knockdown of some genes, such as kinesin-5 or kinesin-7-II subfamily genes (Miki et al. 2014, Naito and Goshima 2015). In addition, we observed a specific defect in nuclear positioning in the RNAi lines corresponding to the kinesin-ARK subfamily (Fig. 3; Supplementary Fig. S2).

Fig. 1 The nucleus is translocated to the cell center in a microtubule (MT)-dependent manner after cell division in apical cells. (A–C) Time-lapse imaging (wide-field microscopy) of caulonemal cells expressing GFP–tubulin (green) and histone H2B–mRFP (red). The MT-destabilizing agent oryzalin (10 μM), actin-destabilizing drugs, latrunculin B (25 μM) or cytochalasin B (200 μM), or control (0.5% or 1.0% DMSO) were added to telophase cells at 5–6 min (each group: 2 ml of the solution). Images were acquired every 1 min. Time 0 corresponds to the metaphase–anaphase transition. Dotted lines indicate cell tips at 10 and 90 min. See also Supplementary Movie S2. Scale bar = 100 μm. (D) The location of the nucleus before and after drug treatment in apical cells. The distance from the metaphase plate (the center of the chromosome mass at the metaphase) was plotted as the mean ± SEM (DMSO, n = 7; oryzalin, n = 7; latrunculin B, n = 6; cytochalasin B, n = 7).
Kinesin-ARK is a plant-specific subfamily and, in *P. patens*, four highly homologous genes (*kinesin-ARK-a–d*) belong to this subfamily ([Supplementary Figs. S2A, S3](#)). Each member of the kinesin-ARK subfamily has a motor domain at the N-terminus and the armadillo repeat motif near the tail region ([Supplementary Fig. S2B](#)). In Arabidopsis, a loss of kinesin-ARKs (called ARK1, ARK2 and ARK3) increases MT numbers in root hairs and impairs morphology of root hair cells (Jones et al. 2006, Yang et al. 2007, Sakai et al. 2008). Our previous localization study suggested that kinesin-ARK-a and -b are strongly expressed in protonemal cells, but we detected little or no expression of kinesin-ARK-c or -d, respectively (Miki et al. 2014). During mitosis, kinesin-ARK-a and -b are located along the spindle and phragmoplast MTs (Miki et al. 2014). Kinesin-ARK-a and -b are highly homologous to each other even at the nucleotide sequence level, suggesting that they function redundantly and that an RNAi construct designed to target one of them is expected to knock both genes.

![Fig. 2 The flowchart of the kinesin RNAi screening.](#)

In total, we selected > 450 RNAi candidate lines in which 61 genes were targeted with 101 constructs. Time-lapse imaging of the GFP–tubulin, histone H2B–mRFP and chloroplasts (autofluorescence) was performed using wide-field microscopy. Scale bars = 10 μm (a metaphase cell), 2 cm (6-well plate) or 100 μm (screened cells).
down [this was the case for several mitotic genes (Nakaoka et al. 2012, Kosetsu et al. 2013, Miki et al. 2014)]. We have selected six independent stable RNAi lines corresponding to three constructs that targeted two non-overlapping regions in the kinesin-ARK genes (Supplementary Fig. S2C). Quantitative real-time reverse transcription–PCR (qRT–PCR) analysis confirmed that each construct down-regulated kinesin-ARK-a and -b simultaneously (Supplementary Fig. S2D). The mRNA level was reduced by >40% in most RNAi lines, with some variability, which is typical of our inducible RNAi system (Nakaoka et al. 2012, Kosetsu et al. 2013, Miki et al. 2014).

An analysis of the time-lapse movies of the six RNAi lines did not identify the defects in tip growth or mitotic duration (Supplementary Fig. S4A, B). The overall growth of the protonemata and development of the gametophore, a shoot of moss, were also unaffected (Supplementary Fig. S4C). However, the caulonemal cells of these lines exhibited a characteristic phenotype (Fig. 3; Supplementary Movie S4).

Mitosis proceeded in exactly the same way as in the control cells, but the daughter nucleus in the apical cell moved back to the cell plate at a very high frequency at the rate of 0.34 ± 0.15 μm min⁻¹, while it moved forward in control cells at the rate of 0.31 ± 0.02 μm min⁻¹ (mean ± SD, between 54 and 81 min in Fig. 3B). The nucleus remained near the cell plate for the next few hours in the RNAi-treated cells.

We investigated the possible effect of actin or MT on the observed backward movement of the nucleus by live cell imaging, combined with the addition of drugs. When the cells were treated with latrunculin B 20–21 min after anaphase, the daughter nucleus of apical cells remained in the control cells. However, they moved back to the cell plate in the absence of kinesins (Fig. 4A, B, E; Supplementary Movie S5). This behavior was identical to that exhibited by untreated cells (Fig. 3A, B). In sharp contrast, when the cells were treated with oryzalin, the nucleus in the tip cells remained at the same position in both kinesin-ARK RNAi and the control cells (Fig. 4C, D, E; Supplementary Movie S5). Therefore, we concluded that the backward movement was dependent on MTs, but not on actin.

Fig. 3 Kinesins of the ARK subfamily (armadillo repeat-containing kinesins) are necessary for nuclear translocation at the end of cell division. (A) Time-lapse imaging (wide-field microscopy) of caulonemal cells expressing GFP–tubulin (green) and histone H2B–mRFP (red). The representative control and kinesin-ARK RNAi cells (first construct, line #7) and the kymographs (A') are displayed. Images were acquired every 3 min. Time 0 corresponds to the start of sister chromatid separation. Horizontal bars = 100 μm; vertical bar = 30 min. See also Supplementary Movie S4. (B) Nuclear movement at the end of cell division in control and kinesin-ARK RNAi apical cells (n = 5 each group, mean ± SEM). The backward movement of the nucleus during 20–40 min in the control cells in this experiment is slightly different from that of the control cells presented in Fig. 1. We think that this reproducible difference is due to a difference in culture conditions; in Fig. 1, 0.5 ml of the medium was added to the cells cultured in the agar medium during the course of imaging, whereas in this figure the cells were examined without addition of the extra medium (however, water was added prior to imaging in order to avoid dryness in the agar). (C) The frequency of the nuclear translocation defect after kinesin-ARK RNAi [kinesin-ARK RNAi first construct (line #7, n = 25; line #8, n = 15), second construct (line #7, n = 43; line #9, n = 33), third construct (line #2, n = 16; line #3, n = 27), control (n = 75)]. For each cell division event, we assessed whether the daughter nucleus in the apical cells moved back to the cell plate.
Abnormal migration of the nucleus in the absence of kinesin-ARK depends on MTs, but not on actin. (A–D) Time-lapse imaging (wide-field microscopy) of caulonemal cells expressing GFP–tubulin (green) and histone H2B–mRFP (red). The actin-destabilizing drug latrunculin B (25 μM) or the MT-destabilizing drug oryzalin (10 μM) was added 20–21 min after the onset of anaphase in the control or kinesin-ARK RNAi cells. Images were acquired every 1 min. Time 0 corresponds to the metaphase–anaphase transition. Scale bar = 100 μm. See also Supplementary Movie S5. (E) The location of the nucleus before and after drug treatment in the apical cells. The distance from the metaphase plate (the center of the chromosome mass during the metaphase stage) was measured for multiple RNAi lines. Since the nucleus in all RNAi transgenic lines exhibited similar behaviors, we combined the data set, and plotted the nuclear position as mean ± SEM [control + latrunculin B, n = 5; kinesin-ARK RNAi + latrunculin B, n = 6 (RNAi second construct line #7, n = 3; line #9, n = 2; third construct line #2, n = 1); control + oryzalin, n = 4; kinesin-ARK RNAi + oryzalin, n = 4 (RNAi second construct line #7, n = 1; line #9, n = 3)].

Fig. 4
In RNAi-treated cells, we observed renewed forward movement in the cell plate-proximal nucleus, towards the cell center $92 \pm 34$ min ($\pm$ SD, $n = 5$) prior to the subsequent mitotic phase at the rate of $0.58 \pm 0.45$ $\mu$m min$^{-1}$ (Supplementary Movie S3; a cell indicated by a white arrow around 5 h 45 min). However, the nuclear position was not fully restored, and we noticed that the mitotic spindle in the RNAi-treated cells was formed at greater proximity to the cell plate, as compared with the control cells (Supplementary Fig. S5).

Therefore, the ARK subfamily of kinesins and MTs are necessary for nuclear centering, specifically after cell division, which affects the cell division site of subsequent mitosis.

Kinesin-ARCKs are associated with MT bundles surrounding the moving nucleus

When high-resolution imaging was performed using confocal microscopy, we identified bundled MTs attached to the moving nucleus in four of five control cells (Fig. 5A, arrowheads; Supplementary Movie S6).
Supplementary Movie S6). Such MT bundles, however, were not observed in the RNAi cells (0 of 4 cells). These results suggested that kinesin-ARK protein as a linker connects multiple MTs to the nucleus.

We determined localization of kinesin-ARK-a at the time of nuclear translocation. When the Citrine fusion protein was photographed with mCherry–tubulin, we saw that kinesin-ARK-a–Citrine was co-localized with MTs, including those around the nucleus, after chromosome separation (Fig. 5B; Supplementary Movie S7).

Recently, Eng and Wasteney (2014) reported that Arabidopsis ARK1–GFP tracks the growing MT plus ends in several cell types. Furthermore, ARK1 mutant cells showed reduced MT growing velocity and catastrophe frequency in root hairs. Previous studies in Arabidopsis also showed that the growth of the root hair tip is defective due to abnormal abundance of MTs in the endoplasm (Sakai et al. 2008, Yoo et al. 2008). The nuclear migration, however, has not been assessed in those mutants. To verify if these results observed in Arabidopsis ARK1 would hold true for P. patens orthologs, we observed their localization and dynamics by oblique illumination fluorescence microscopy. This method allowed clear visualization of individual MTs near the cell membrane because of the reduced background signals (Konopka and Bednarek 2008, Tokunaga et al. 2008, Nakaoka et al. 2015) (note that the nucleus and the surrounding MTs could not be observed because they were located too far away from the cell cortex). This method confirmed that kinesin-ARK-a–Citrine and kinesin-ARK-b–Citrine are localized along growing and shrinking MTs in the cytoplasm, and are not limited to the growing plus ends (Supplementary Fig. S6; Supplementary Movie S8). We observed that the Citrine signals, which may have corresponded to the clustered kinesins, did not move along the MT (the microscope used was not sensitive enough to detect a single Citrine molecule in vivo). Time-lapse imaging of the GFP–tubulin indicated no changes in the MT growth rate in kinesin-ARK RNAi cells (Supplementary Fig. S7A). Due to the growth of the MTs beyond the imaging field, we were unable to measure the catastrophe frequency. However, we also observed, through immunofluorescence microscopy of the MTs, that the overall MT abundance remained unaltered in the absence of kinesin-ARK (Supplementary Fig. S7B).

These results suggest that kinesin-ARK plays little role in the regulation of MT dynamics in protonemal cells. This situation could be due to an inefficient knockdown of kinesin-ARKs in the system, or differences among the cell types. Alternatively, Arabidopsis ARKs might have acquired a new function during evolution.

Kinesin-ARK-a possesses a plus-end-directed motor activity

The motor activity of kinesin-ARKs has not been characterized in previous studies. To test whether P. patens kinesin-ARKs have the plus-end-directed motor activity, we performed an MT gliding assay using the purified truncated kinesin-ARK-a that possesses the motor and dimerization domains (amino acids 64–482; Fig. 6A, B; the domain organization is highly conserved among kinesin-ARK-a–d). In this assay, kinesin motors tagged with GFP were bound to the glass surface via an anti-GFP antibody, and fluorescently labeled MTs near the glass surface were examined under a total internal reflection fluorescence microscope (TIRFM). In the presence of ATP, we observed MT gliding at the rate of 11.7 ± 7.1 μm min⁻¹ (~200 nm s⁻¹; mean ± SD), whereas the motion was not observed when ATP was replaced with AMP-PNP, a non-hydrolyzable analog of ATP (Fig. 6C; Supplementary Movie S9). Finally, to assess directionality of the motor, we utilized polarity-marked MTs in a gliding assay. The imaging data indicated that the kinesin-ARK-a motor exhibited plus-end-directed motility exclusively (n = 42 MTs; Fig. 6D, E). We concluded that kinesin-ARK-a is an MT plus-end-directed motor protein.

Discussion

This study is focused on nuclear positioning, an intracellular process generally believed to be actin dependent in plant cells (Chytirilova et al. 2000, Iwabuchi et al. 2010, Tamura et al. 2013). Our data demonstrate that MTs and an MT-based motor protein—the kinesin-ARK subfamily—are necessary for proper positioning of the nucleus in the tip-growing protonemal apical cells of the moss P. patens (Fig. 7). The strongest evidence supporting this notion is that the centering of a daughter nucleus at the end of cell division is perturbed by either an MT destabilizing drug or an RNAi knockdown of a potent motor protein, kinesin-ARK. Nuclear translocation in other phases is also shown to require MTs, but not kinesin-ARKs. On the other hand, our results suggest that actin and myosin play little role in the nuclear positioning process in these cells.

Mechanism of kinesin-ARK-mediated nuclear positioning

How does kinesin-ARK drive nuclear positioning? One possibility is that kinesin-ARK works rather indirectly. The tail domain of Arabidopsis ARK is known to bind to the NIMA-related kinase in vitro (Sakai et al. 2008) and ARK1 genetically interacts with AGD1, a class I ADP ribosylation factor GTPase-activating protein (ARF-GAP) (Yoo et al. 2008, Yoo and Blancaflor 2013). These signaling molecules might control nuclear positioning via an unknown mechanism. Alternatively, kinesin-ARK may regulate MT dynamics and thereby alter the pushing/pulling force of MTs applied to the cell plate; this mechanism might in turn affect forces acting on the nucleus, which associates with the MTs. However, we did not observe aberrations in MT dynamics or MT numbers. Therefore, while the above explanations cannot be ruled out, perhaps the most likely scenario is the following: the kinesin-ARK protein transports the nucleus as a cargo, just like kinesins and dynesin in animal cells and myosin Xl in Arabidopsis do. The plus-end-directed motor activity of kinesin-ARK and the MT orientation in the apical cell are consistent with this scenario. In protonemal apical cells, MTs are aligned longitudinally throughout the cytoplasm (Doonan et al. 1985). The majority of growing plus ends move to the apical side in the
apical region of the cells, whereas MTs display a mixed polarity in the basal region (i.e. behind the nucleus) (Hiwatashi et al. 2014). An efficient way to transport the nucleus to the apical side would be to use the plus-end-directed kinesins on the uniformly oriented MTs. In this scenario, it is expected that MTs are laterally associated with the nuclear surface during the migration. Indeed, around the migrating nucleus, we frequently observe MT bundles that form in a kinesin-ARK-dependent manner, and the motor protein is associated with those bundles. The key to proving this mechanism is to identify the adaptor protein that bridges the NE and the kinesin-ARK motor.

**Kinesin-ARK is localized along the endoplasmic MT**

We have demonstrated that the kinesin-ARK motor protein has an MT gliding activity in vitro. In protonemal cells, it was localized on MTs. However, we did not detect motility of the punctate kinesin-ARK–Citrine signals along the MT near the cell cortex. Lack of motility in vivo may be due to this kinesin having a non-processive motor domain (i.e. performing only one step before dissociation). Alternatively, the kinesin-ARK identified on the MTs may be present in an inactive form, and motility may not be observed unless a cargo binds to the kinesin. In fact, the cargo-dependent activation is a commonly observed characteristic of kinesin family proteins (Verhey and Hammond 2009). If the nucleus were the cargo of kinesin-ARK, the non-motility of this motor near the cortex would make sense, as the nucleus is located more towards the interior of the cell. However, Citrine fusion to the kinesin attenuating functionality is a possibility that cannot be excluded, although Arabidopsis ARK1 was proven to be functional despite GFP tagging at the C-terminus (Eng and Wasteneys 2014).

**Nuclear positioning in the protonemal apical cell cycle**

Our data are also suggestive of the presence of four force-generating mechanisms acting on the nucleus in apical cells. First, we observe forward movement during cytokinesis (Fig. 3B, 3–21 min; Fig. 7, phase I). This movement is MT dependent, since it was inhibited when oryzalin was added to the telophase cell (Fig. 1). Secondly, the nucleus migrates a short distance towards the cell plate (Fig. 3B, 24–51 min; Fig. 7, phase II). This movement was also dependent on MTs, but independent of actin (Fig. 4). Considering the MT orientation in the apical cell (Hiwatashi et al. 2014), a possible explanation is that the nucleus is transported by a minus-end-directed kinesin. Thirdly, the nucleus is moved to the center of the cell by the action of MTs and kinesin-ARKs, which might transport the nucleus as a cargo (Fig. 3B, 54–81 min; Fig. 7, phase III). If this were the case, however, we might also postulate the presence of opposing motors or microtubule-associated proteins on the nucleus and/or cytoplasmic viscoelasticity, since the rate of nuclear migration was much lower than the MT gliding velocity.
of the kinesin-ARK motor domain. Finally, we found that a nucleus that is aberrantly located near the basal side—in the absence of kinesins of the ARK subfamily—eventually migrates, albeit not completely, to the middle region of the apical cell before subsequent mitosis (Supplementary Movie S3; Fig. 7, phase IV). This pattern of migration is reminiscent of that observed in subapical cells, where the MT-dependent mechanism places the nucleus at the neck of side branches prior to mitosis (Doonan et al. 1986). An unidentified plus-end-directed kinesin or global reorganization of MTs might be involved in this type of migration.

Conclusions

This study provides a new mechanistic view of nuclear positioning in plant cells; namely, the nucleus may be driven by MT- and kinesin-dependent mechanisms. This finding suggests that the intrinsic mechanisms underlying this process are widely conserved among fungal, animal and plant species; which mechanism predominates depends on the cell type. Indeed, actomyosin systems also contribute to nuclear movement in animal cells (Norden et al. 2009, Gundersen and Worman 2013). Whether flowering plants generally possess a similar MT- and kinesin-dependent mechanism has yet to be determined. Intriguingly, MTs were shown to be necessary for prevention of the actin-dependent mechanism of nuclear migration in the root hair of a legume species (Lloyd et al. 1987). It would be interesting to evaluate the contribution of kinesin-ARK to this and other processes in flowering plants.

Materials and Methods

Moss culture, RNAi and transformation

For moss culture and transformation, we essentially followed the methods described in Nakaoka et al. (2012) and Miki et al. (2014). In brief, BCDAT medium on which cellophane (a gift of Futamura Chemical Industries) was placed was used for regular culturing of protonemata at 25°C under continuous white light. Transformation was performed by the standard polyethylene glycol-mediated method using the protoplast (drielsae (Kyowa Hakko Kogyo) was used for cell wall digestion). To observe RNAi phenotypes, we cultured protonemata for 5–6 d in the presence of 1 μM β-estradiol that turns on the promoter (Kubo et al. 2013) and thereby expression of dsRNAs. Protonemal cells, which had been cultured on a 35-mm glass-bottom dish (MatTek) or 6-well glass-bottom plate (IWAKI) with BCD agar medium at 24–25°C, were imaged. In the RNAi screening, we prepared a control GFP–tubulin/histone H2B–mRFP line and five RNAi lines each time, and imaged a total of approximately 30 sites every 3 min for >6 h.

Plasmids and moss lines

The background strain of all moss lines used in this study was the Gransden 2004 line obtained from M. Hasebe (NIBB, Japan). GFP–tubulin/histone
H2B–mRFP (Nakaoka et al. 2012) and kinesin-ARK–Citrine/mCherry–tubulin lines (Miki et al. 2014) have been previously described. In brief, the GFP–tubulin construct contains the rice actin promoter, sGFP, the β-tubulin gene (AB096718), an rbcS terminator, a G418-resistant cassette and the H78 target site (Hiwatashi et al. 2008). The histone H2B–mRFP construct contains the 7113 promoter (Mitsuhashi et al. 1996), the human HistoneH2B gene, the mRFP gene, the terminator sequence of Agrobacterium tumefaciens nopaline synthase, a zeocin-resistant cassette and the 213 gene targeting site (Nakaoka et al. 2012). The mCherry–tubulin construct contains the rice actin promoter, the mCherry gene, the β-tubulin gene, an rbcS terminator, a hygromycin-resistant cassette and the PIG1 target site (Miki et al. 2014). In order to obtain kinesin-ARK–Citrine lines, integration plasmids were constructed, where the Citrine was fused to the C-terminus of kinesin-ARK. The approximately 1 kb C-terminus and the approximately 1 kb 3'- untranslated region (UTR) sequences of the kinesin-ARK genes were flanked by the Citrine gene, the terminator sequence from A. tumefaciens nopaline synthase and a G418-resistant cassette (Miki et al. 2014, Yamada et al. 2015). Kinesin RNAi constructs were obtained using the Gateway system (Invitrogen) with pGCa626 (Nakaoka et al. 2012) as the destination vector. The probable gene targeting site of pGCa626 is the PIG1 locus, but the actual integration site(s) were not verified. PCR primers used for RNAi are listed in Supplementary Table S1.

Microscopy, drug treatment and immunostaining

We essentially followed the imaging methods described in Nakaoka et al. (2012, 2015), Koestu et al. (2013), Miki et al. (2014), Naito and Goshima (2015) and Yamada et al. (2015). For the imaging of drug-treated cells and long-term imaging of the RNAi lines, fluorescence imaging was performed with a Nikon Ti microscope ([×20, 0.75 NA (drug treatment), ×10, 0.45 NA or ×10, 0.50 NA (RNAi screening) lens] equipped with an electron-multiplying charged-coupled device (EMCCD) camera (Evolve; Roper or IXon3; Andor) or a Nikon TE2000E microscope ([×10, 0.30 NA lens] equipped with an EMCCD camera (Micromax; Roper). Images were acquired every 1 or 3 min (drug treatment) or at multiple sites every 3 min (RNAi screening). For the high-magnification, time-lapse microscopy of the kinesin–Citrine or RNAi lines, the Nikon Ti2000 microscope equipped with the spinning-disc confocal unit CSU-X1 (Yokogawa) and an EMCCD camera (ImagEM; Hamamatsu) was used ([×100, 1.40 NA, ×60, 1.40 NA or ×40, 1.30 NA lens]). A ×1.5 intermediate magnification lens was also used for the observation of MT dynamics. Images were acquired every 3 s (for MT dynamics), 0.5 min or 1 min. For in vivo oblique illumination microscopy (Nakaoka et al. 2015) or in vitro TIRFm assay, the Nikon Ti microscope ([×100, 1.40 NA lens] equipped with an EMCCD camera (Evolve; Roper) and 488/561 nm excitation laser was used. A ×2.5 intermediate magnification lens was also used for the in vivo observation. Images were acquired every 3 s (in vivo) or 1 s (in vitro). The microscopes were controlled by the Micromanager software (Fedelstein et al. 2010) and image data were analyzed with ImageJ. In RNAi screening, we inoculated a piece of the protonemata onto (in vivo) or 1 s (in vitro). The microscopes were controlled by the MEGA5 software (Tamura et al. 2011). Statistical support for internal branches by bootstrap analyses was calculated using 1,000 replications. Kinesin-ARKs of the other species were obtained by a BLASTP search using the amino acid sequence of Physcomitrella patens (Pp) kinesin-ARK-a as the query. The Arabidopsis Information Resource (http://www.arabidopsis.org) and Phytozome (http://www.phytozome.net) (for Selaginella moellendorfii) were used as the database. Gene IDs are as follows. Pp kinesin-ARK-a (PhyPA_455498/ Pp1s280_660V), Pp kinesin-ARK-b (PhyPA_453488/Pp1s242_111V), Pp kinesin-ARK-c (PhyPA_425827/Pp1s14_211V), Pp kinesin-ARK-d (PhyPA_427907/Pp1s12_256V), At Ark-1 (AT3G54870), At Ark-2 (AT1G09105), At Ark-3 (AT1G12430).

Protein purification

The motor and dimerization domains of kinesin-ARK-a were fused with mGFP and 6 x His, and cloned into an Escherichia coli expression vector pET23a (Novagen). Primers listed in Supplementary Table S1 were used for PCR, and cloning was performed with NdeI/NorI/XhoI digestion. The resultant plasmid pGcG886 (kinesin-ARK-a-motor-mGFP-6 x His) was transformed into the E. coli SoluBL2 strain (Genlantis). Proteins were expressed at 18 °C with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The bacterial pellet from 250 ml of culture was homogenized with a homogenizer (Braemor-Emerson Sonifier 250-D Advanced; BRANSON) in lysis buffer (50 mM NaPO4, pH 8.0, 250 mM NaCl, 2 mM MgCl2, 20 mM imidazole, 10 mM 2-mercaptoethanol, 1 mM ATP, protease inhibitors). Post-centrifugation, the supernatant of the bacterial extract was incubated with Ni-NTA resin for 2 h at 4 °C. The Ni-NTA resin was washed with wash buffer (50 mM NaPO4, pH 6.0, 250 mM NaCl, 1 mM MgCl2, 60 mM imidazole, 10 mM 2-mercaptoethanol, 0.1 mM ATP) followed by purification with Ni-NTA resin and elution buffer (50 mM NaPO4, pH 7.2, 250 mM NaCl, 1 mM MgCl2, 500 mM imidazole, 10 mM 2-mercaptoethanol, 0.1 mM ATP). The buffer was exchanged to BR880 (80 mM K-PiPES, pH 6.8, 2 mM MgCl2, 1 mM EDTA) containing 10 mM 2-mercaptoethanol and 0.1 mM ATP by PD MiniTrap G-25 (GE Healthcare). The protein was further affinity-purified using taox-stabilized MTS and 1 mM AMP-PNP. The mixture was centrifuged at 80,000 × g for 15 min at 25 °C, followed by release of the active motor with 5 mM ATP. The MT gliding assay was performed immediately after protein purification.

MT gliding assay

The conventional MT gliding assay (Woehlke et al. 1997) was performed with some modifications. Flow chambers were assembled between a siliconized coverslip and a microslide glass with double-sided sticky tape. The coverslip was coated with the anti-GFP antibody (rabbit polyclonal, affinity-purified). The chamber was washed with BR880 and purified GFP-tagged kinesin-ARK-a motor was flowed into the chamber. After washing with BR880 containing 0.5 mg ml−1 k-casein, the motility buffer (BR880, GMP-CPP-stabilized MTS with Alexa 568-labels, 2 mM ATP or AMP-PNP, 0.5 mg ml−1 k-casein and 0.1% methylcellulose), 20 μM taxol with an oxygen scavenger system [50 mM glucose, 400 μM glutathione, 200 μg ml−1 catalase and 4 mM diethiothreitol (DTT)] (Li et al. 2012) was flowed into the chamber. For polarity-marked and GMP-CPP-stabilized MTs preparation, bright MTs seeds (80% unlabeled tubulin: 20% Alexa 568-labeled tubulin) were first assembled, followed by polymerization of dim MTs [67% unlabeled tubulin: 2% Alexa 568-labeled tubulin: 31% N-ethylmaleimide (NEM) tubulin]. Imaging was performed with a TIRFM, and the gliding velocity was measured with kymographs.
Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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