Armadillo repeat-containing kinesin represents the versatile plus-end-directed transporter in plants

Mari W. Yoshida¹, Maya Hakozaki ¹, and Gohta Goshima¹,²*

¹ Department of Biological Science, Graduate School of Science, Nagoya University, Nagoya 464-8602, Japan
² Sugashima Marine Biological Laboratory, Graduate School of Science, Nagoya University, Toba 517-0004, Japan

*To whom correspondence should be addressed. Email: goshima@bio.nagoya-u.ac.jp

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Abstract

Kinesin-1, also known as conventional kinesin, is widely utilised for microtubule plus-end-directed (“anterograde”) transport of various cargos in animal cells. However, a motor functionally equivalent to the conventional kinesin has not been identified in plants, which lack the kinesin-1 genes. Here, we show that plant-specific armadillo repeat-containing kinesin (ARK) is the long-sought-after versatile anterograde transporter in plants. In ARK mutants of the moss Physcomitrium patens, the anterograde motility of nuclei, chloroplasts, mitochondria, and secretory vesicles was suppressed. Ectopic expression of non-motile or tail-deleted ARK did not restore organelle distribution. Another prominent macroscopic phenotype of ARK mutants was the suppression of cell tip growth. We showed that this defect was attributed to the mislocalisation of actin regulators, including RopGEFs; expression and forced anterograde transport of RopGEF3 suppressed the growth phenotype of the ARK mutant. The mutant phenotypes were partially rescued by ARK homologues in Arabidopsis thaliana, suggesting the conservation of ARK functions in plants.

Key words

Kinesin/Microtubule-based transport/Cell tip growth/Actin/Physcomitrium (Physcomitrella) patens

Introduction

The proper positioning of cellular components in the cytoplasm is critical for cell physiology. It is particularly important for highly polarised cells, such as neurons and epithelial cells, in animals. Regulated bidirectional transport on microtubules is a major mechanism for positioning intracellular components and requires motor proteins that bind to cargo and walk on microtubules¹–³. In animal cells, cytoplasmic dynein is responsible for the majority of minus-end-directed (retrograde) transport in the cytoplasm, and a few kinesin family members have been identified as plus-end-directed (anterograde) transporters¹. Among them, kinesin-1, also called conventional kinesin, is a universal transporter whose critical roles have been identified in various cell types⁴–⁶. Cargos of kinesin-1 include giant organelles, such as nuclei and mitochondria, as well as smaller
cellular components, such as secretory vesicles, protein complexes, and RNA granules. These anterograde and retrograde transporters work together on the same cargo to allow bidirectional transport.

Intriguingly, plants do not possess cytoplasmic dynein or conventional kinesin. Intracellular transport in plants has long been believed to be predominantly driven by myosin and actin. Cytoplasmic streaming is the best-studied phenomenon observed in many plant cell types and is powered by myosin motors. Furthermore, in Arabidopsis thaliana root hair, myosin XI-i transports the nucleus to the centre of the growing cell. Chloroplast photo-relocation, a phenomenon in which chloroplasts are repositioned in response to light stimuli, is also promoted by specific chloroplast-associated actin filaments that polymerise between the plasma membrane and the chloroplasts in Arabidopsis thaliana. However, the microtubule-dependent motility of cellular components also exists in plants. For example, nuclear movement requires microtubules in meristemoid mother cells in Arabidopsis thaliana and microspores in Nicotiana tabacum. In the former case, nuclear positioning affected stomatal patterning, suggesting the physiological importance of microtubule-dependent nuclear migration. A series of studies using protonemal tissue of the moss Physcomitrium patens (formerly called Physcomitrella patens) have also revealed the contribution of microtubules and motors to organelle transport. The tip-growing apical cell of Physcomitrium patens protonema is an ideal system for studying microtubule-dependent bidirectional transport, as ~90% of the microtubules are oriented in such a manner that the plus ends face the cell tip during interphase. In this system, when plus-end-directed motility is predominant over minus-end-directed motility, the organelle moves apically and is abundant on the apical side, and vice versa. Based on this advantage, several kinesin-14 family members have been identified as the drivers of retrograde transport, namely KCH for the nucleus, KCBP for the chloroplast and telophase chromosome, and ATK for newly-formed microtubules. Studies in Physcomitrium patens thus drew a functional analogy between kinesin-14s and dynein and further illustrated the contribution of intracellular transport driven by the microtubule-kinesin mechanism.

Anterograde transporter is a missing piece in the model of intracellular transport in plants. Plant genomes do not encode proteins paralogous to kinesin-1, which is characterised by a unique domain in its tail that binds to the light chain. Kinesin-3 is another potent cargo transporter in animal cytoplasm, and it is best known as a synaptic vesicle transporter in neurons. However, this subfamily is also missing in plants; thus, no deduction can be made from genome sequences regarding the identity of the versatile anterograde transporter in plants. Nevertheless, armadillo repeat-containing kinesin (ARK), which constitutes a subfamily unique to the plant lineage, could be a candidate motor protein. In a previous study, we observed that the nucleus was not properly positioned after inducing RNAi that targeted two of the four ARK genes in moss protonemata. However, whether ARK is a genuine transporter of the nucleus could not be clarified. Although the purified ARKb motor can glide microtubules in the conventional in vitro gliding assay (i.e. motor activity is present), the motility of ARKb-Citrine on the microtubules in vivo could not be observed. This contrasts with the observation that retrograde transporters show fast (~400 nm/s) and long (~1 μm) minus-end-directed motility in vivo. Furthermore, AtARK1, the most-studied ARK, is localised to the plus end of microtubules, triggers microtubule catastrophe, and tethers...
the ER to the microtubule end by binding to the ER-shaping protein atlastin GTPase RH3. Alterations in microtubule dynamics can, in principle, affect nuclear motility, as shown in yeast. However, none of these previous studies have clarified the mechanism by which ARK positions nuclei.

This study initially aimed to elucidate how ARK proteins position the nuclei. Surprisingly, in the newly established ARK mutant lines, microtubule plus-end-directed motility of not only the nuclei but also chloroplasts, mitochondria, and secretory vesicles was suppressed. Ectopic expression of a non-motile mutant or tail-deleted ARK did not restore organelle distribution. Furthermore, when a brighter fluorescent protein, mNeonGreen (mNG), was attached to the endogenous ARKb, processive motility towards the microtubule plus ends was observed in vivo. The most prominent macroscopic phenotype of ARK mutants was the suppression of cell tip growth, which is known to require the actin cytoskeleton. Interestingly, this defect was attributed to the mislocalisation of actin regulators, including the guanine nucleotide exchange factors (GEFs) of Rho-type GTPases. These phenotypes were partially rescued by the ectopic expression of ARK homologues in A. thaliana. The results suggest that ARK is a conserved and versatile anterograde transporter in plants.

Results

P. patens ARK is required for protonemal growth

Four highly homologous genes have been identified in the ARK family of moss (Fig. 1A). Previously, we examined the RNAi knockdown mutant of ARK and identified a specific defect in protonemal apical cells, namely, nuclear mispositioning. The RNAi targeted ARKa and ARKb genes and reduced their mRNA levels by ~50%, whereas ARKc and ARKd were kept intact, which might have prevented the expression of several potential phenotypes. To isolate multiple loss-of-function mutants of ARK, we used CRISPR/Cas9. We attempted to simultaneously introduce a frameshift just before the critical, conserved ATPase motif or microtubule-binding site in the motor domain of ARKa-d, which would destroy its motor activity. We used two parental lines, which expressed mCherry-α-tubulin and GFP-α-tubulin/Histone2B-mCherry. Consequently, we obtained independent mutants containing a variety of mutations in different gene sets from both backgrounds. Overall, all the isolated single- and double frame-shifted lines (a, b, ab, ad, bc, and bd) showed no or only mild growth defects (Fig. S1A). In contrast, the colony sizes of the triple (abc) and quadruple (abcd) mutants were dramatically smaller than those of the parental line (Fig. 1B, C, S1A). One line, called ΔARKabcd, possessed frameshift mutations in all the ARK genes (GFP-α-Tubulin/Histone2B-mCherry background), whereas the other, ΔARKabc, had deletions in ARKa, ARKb, and ARKc but ARKd remained intact (mCherry-α-tubulin background) (Fig. S1B). These two lines were chosen for further analyses.

The protonemal tissue was underdeveloped in ΔARKabc and ΔARKabcd. The phenotype was more severe in ΔARKabcd than ΔARKabc, and gametophore tissue (leafy shoot) predominated in the colony (Fig. 1B, top row). The morphology of the ΔARKabc gametophore was normal, but twisted leaves consisting of abnormally shaped cells were observed in ΔARKabcd (Fig. 1B, third and fourth rows). In addition, ΔARKabc and ΔARKabcd developed fewer and shorter rhizoids (root-like filamentous outgrowths) than
the parental lines (Fig. 1B, red arrowheads).

To confirm that the observed phenotype was derived from the disruption of the ARK genes, we performed a rescue experiment in which the ARKb-mNeonGreen (mNG) transgene was ectopically expressed in the mutants. We failed to transform the construct into ΔARKabcd in multiple attempts; sufficient numbers of viable protoplasts for transformation could not be obtained from this extremely unhealthy line. In contrast, ΔARKabc was transformable and a transgenic line stably expressing ARKb-mNG was established. The colony growth of this line was restored, indicating that the protonemal growth defect was caused by a reduction in ARK protein levels (Fig. 1B, C). To determine whether the growth defect was associated with the motor activity of ARKb, we constructed a "rigor" mutant of ARK (ARKb (T169N) -mNG), which binds to microtubules but does not show motility 35. We observed that protonemal colony growth was not rescued by the expression of this mutant ARK (Fig. 1B, C). These results indicate that ARKs with intact motor activity are, likely redundantly, required for protonemal and rhizoid growth as well as gametophore development.

ARK is required for intracellular transport of the nucleus, chloroplast, mitochondrion, and secretory vesicle

We next performed time-lapse fluorescence microscopy on the ARK mutants, focusing on nuclear motility. The underdevelopment of the protonemal tissue prevented us from imaging and analysing the long-distance motility of organelles in ΔARKabcd. Therefore, ΔARKabc which showed less severe protonemal growth defects, was used for the cellular analysis. Consistent with the results of a study using ARKab RNAi 27, the nucleus moved basally after anaphase and was located adjacent to the basal cell wall in the apical cells of ΔARKabc (Fig. 2A, B; nuclei can be identified as an area devoid of mCherry-a-tubulin signals in each z-slice). Like the control cells, the mispositioned nuclei in the mutant cells also showed apical movement during mitotic prophase (Fig. 2B; -60–0 min). However, because of the overly basal localisation of the nucleus during interphase, mitotic spindle formation occurred more basally than in the control line (Fig. 2C). The mispositioning of the spindle was more drastic than that observed in the previous ARKa/b RNAi line 27, suggesting that the previous RNAi line represented a weaker hypomorphic allele. Importantly, the position of the nucleus and spindle was largely rescued by ectopic expression of ARKb-mNG but not by that of the rigor mutant, indicating that the nuclear mispositioning was associated with the loss of ARK motor activity (Fig. 2B, C).

The constant, rapid, and bidirectional motility of chloroplasts during the normal cell cycle is dependent on microtubules, and KCBP kinesin (kinesin-14VI) drives their retrograde transport 22. However, the factor(s) responsible for anterograde movement (i.e. plus-end-directed transporters) remains unknown. During imaging, we realised that chloroplasts in the mutant behaved differently from those in the control line. In ΔARKabc, chloroplasts moved basally overall and clustered near the basal cell wall in the apical cell (Fig. 2A, D, E, Movie 1). This behaviour was opposite to that of the KCBP knockout line, in which chloroplasts were predominantly moved to and observed on the apical side of the cell 22. This phenotype was also rescued by ectopic expression of ARKb-mNG but not by the rigor mutant (Fig. 2A, E). These results indicate that the bidirectional motility of the chloroplast is driven by KCBP (retrograde) and ARK (anterograde).
To the best of our knowledge, ARK is the first anterograde kinesin to be identified as a participant in the movement of multiple organelles in plant cells. This led us to hypothesise that ARK may be a versatile anterograde motor for many cargos, similar to conventional kinesin in animals. To test this possibility, we analysed the mitochondrion, another giant membranous organelle possessing DNA. To visualise the mitochondria, the mitochondrial membrane protein γ-F1ATPase-mNG was expressed in wild-type and ΔARKabc (note that ΔARKabcd is not amenable to transformation). Mitochondria showed bidirectional movement along cytoplasmic microtubules; however, most of them translocated apically from their initial positions in the control line after tracking for 6 min (65 of 72 mitochondria in six cells; Fig. 3G). In contrast, more basal translocation was observed in ΔARKabc (38 of 72 mitochondria in six cells). These results suggest that ARK is involved in the plus-end-directed transport of mitochondria.

Next, we focused on secretory vesicles, which are also known cargos of kinesin-1 and kinesin-3 in animals. In *P. patens*, actin-based motor myosin XI transports RabE14-positive vesicles near the cell apex. Whether microtubules and kinesins are involved in vesicle transport remains unknown. Referring to the literature on *Arabidopsis*, we labelled vesicles by tagging endogenous RabA2b with mNG in the wild-type and ΔARKabc lines. RabA2b is a small GTPase homologous to Rab-A2 in *A. thaliana*, which regulates the secretory pathway by recruiting SNARE proteins. In the control line, the mNG-RabA2b signal accumulated at the apex (Fig. 3A). Time-lapse imaging using a spinning-disc confocal microscope identified motile small puncta. When we focused on long-distance movement of the puncta toward the cell tip (i.e. plus-end-directed motility), we observed that it was markedly suppressed when the microtubules or actin were depolymerised by latrunculin A or oryzalin (Fig. 3C). Thus, both actin- and microtubule-dependent mechanisms are responsible for the long-range transport of RabA2b-containing vesicles. In ΔARKabc, the number of long-distance movements of mNG-RabA2b slightly decreased, whereas ectopic expression of ARKb-mNG restored motility (Fig. 3D). Consistent with these observations, the RabA2b signal at the apex was smaller in ΔARKabc than the control line (Fig. 3A, B). These results suggest that ARK is involved in the microtubule plus-end-directed transport of RabA2b-containing vesicles, in addition to giant organelles.

**Microtubule polymerisation dynamics are unaltered in the absence of ARK in moss**

*Arabidopsis* ARK1 promotes microtubule catastrophe in root hair cells. To examine whether this holds true for moss ARK in protonemata, we used oblique illumination fluorescence microscopy to trace individual microtubule ends in protonemal apical cells in the mutants. Most of the parameters of microtubule dynamics, including catastrophe frequency, were not significantly altered in ΔARKabc or ΔARKabcd (Fig. S2A, Table S1). The growth rate was slightly increased in ΔARKabc but not in ΔARKabcd. Microtubule orientation near the cell tip was further analysed through tracing EB1-Citrine, which binds to the growing ends of microtubules. Similarly, drastic difference was not detected between ΔARKabc and control (Fig. S2B). Thus, the observed phenotype of the mutants is unlikely to be due to the misregulation of polymerisation dynamics or the misorientation of the microtubules.

**ARK shows processive, plus-end-directed motility in vivo**
Although endogenous ARKs labelled with Citrine were localised to the microtubules, directional movement was not detected in a previous study 27. However, the observed lack of motility might have been due to the insufficient sensitivity of the microscopy, which could not detect a single Citrine molecule 40. In the present study, the brighter fluorescent protein mNG 41 was used to label endogenous ARKb and its localisation was revisited.

Consistent with the previous study, ARKb-mNG was observed on microtubules. Most of them were dissociated from microtubules without showing motility (Fig. 4A, C, Movie 2). Interestingly, however, some small punctate signals moved along the microtubules at 572 ± 243 nm/s (velocity, ± SD) for 986 ± 416 nm (run length, ± SD) (Fig. 4B–D). These values are comparable to those of retrograde transporters (kinesin-14s) in moss (KCBPb, ~413 nm/s, ~1.0 µm; KCHA, ~441 nm/s, ~1.6 µm) 21,28 but much faster than microtubule growth (~100 nm/s; Fig. S2). In contrast, no movement was observed in the rigor mutant ARKb (T169N) -mNG (Fig. 4C). These results suggest that moss ARK is capable of transporting cargo in vivo.

Tail region of ARK is required for organelle distribution and protonemal growth

ARK family kinesins are distinguished from other kinesins by the presence of an armadillo (ARM) repeat in the tail region, which is known as a protein-protein interaction motif 42. The ARM domain in the tail region is dispensable for AtARK1 to regulate microtubule catastrophe 43. However, if ARK is a cargo transporter, the tail would be a potential cargo-binding site, and therefore, critical for its function. To test whether ARM is required for ARK functions, two deletion constructs were transformed into ΔARKabc (Fig. S3A). Interestingly, the constructs lacking ARM (ΔARM, ΔTail) failed to rescue protonemal tissue growth or the position of the chloroplasts (Fig. S3B, C). Thus, not only motor activity, but also ARM, is indispensable for ARK function in moss.

ARK enables polarised cell growth by localising RopGEF to the cell tip

A prominent phenotype associated with ARK deletion is the suppression of protonemal tissue growth (Fig. 1). At the cellular level, we observed that the protonemal apical and subapical cells were shorter in the mutants than the control line (Fig. 5A, B). One possible explanation for this phenotype is the shortening of the cell cycle (i.e. precocious entry into the M-phase) 44. However, this was not the case, because the duration of the cell cycle was unchanged between the two rounds of mitosis in ΔARKabc (Fig. S4A). Instead, tip elongation significantly slowed in ΔARKabc, which was consistent with the prevalence of shorter cells (Fig. 5C). Furthermore, protonemal cells in ΔARKabcd exhibited round morphology, suggesting that cell polarity was disturbed (Fig. 5A, right). To analyse this phenotype in more detail, we introduced an inducible RNAi construct targeting ARKd into ΔARKabc and observed tip growth upon RNAi induction. Unlike the ΔARKabcd cells, the protonemal cells of this line were rod-shaped before RNAi induction, such that the requirement of ARK for polarised growth could be directly assessed by time-lapse imaging after RNAi induction. We observed that the protonemal cells frequently produced multiple tips in the RNAi line (Fig. S5A, B).

The slowing or suppression of tip growth in protonemal cells is reminiscent of the results of actin dysfunction (confirmed in Fig. S4B) 45,46. Indeed, F-actin foci at the cell apex, which ensure rapid tip growth (Wu and Bezanilla, 2018), were less frequently observed in the absence of ARKabc (Fig. 5D, E). We investigated whether the actin
phenotype could be explained within the framework of microtubule-based transport
required by ARK. In animals, proper actin organisation and dynamics for cell motility
activate the actin polymerisation factor formin [47–49]. The cortical localisation of function-
verified Rop4-mNG [50] was unchanged in the ΔARKabc mutant (Fig. S4C, D).
Interestingly, however, in ΔARKabc we observed a reduction in the cortical signals from
RopGEF3 and RopGEF6, the guanine nucleotide exchange factor (GEF) of ROP, and
For2A, a class II formin that is required for rapid actin elongation and formation of actin
filaments during cell tip growth in moss [51] (Fig. 6A–D). Consistent with these reductions,
oblique illumination microscopy revealed that the growth rate of actin filament near the
tip was reduced by 14% compared to that in the control line (Fig. 5F). Other Rop-GEFs
tagged with mNG were either undetectable or very weakly expressed; therefore, they
were not studied further. The necessity of microtubules for apical enrichment of
RopGEF3 and RopGEF6 was confirmed by oryzalin treatment followed by time-lapse
microscopy (Fig. S4E, F).

We reasoned that if the tip growth defect in the ARK mutant could be attributed to a
defect in the apical transport of actin regulatory molecules, the ectopic expression and
forced apical localisation of RopGEFs would restore the tip growth of the ΔARKabc
mutant. To test this hypothesis, we ectopically expressed RopGEF3-mNG in ΔARKabc.
Colony growth slightly recovered in the transgenic line (Fig. 6H). Next, we constructed
a fusion gene in which tail-deleted ARKb was fused to RopGEF3-mNG and expressed it
in ΔARKabc (Fig. 6E). As expected from a protein with the plus-end-directed motility,
the fusion protein was enriched at the apical cell tip (Fig. 6F, bottom). However, because
the ARM domain was absent in this construct, the chloroplast distribution was not
restored (Fig. 6G). However, the fusion construct substantially restored colony growth
(Fig. 6F, H). Thus, the forced delivery of RopGEF3 to the cell tip using an anterograde
motor was sufficient to restore cell tip growth.

Partial complementation of PpARK mutant by AtARKs
Finally, to gain insight into the evolutionary conservation of ARK function, we
examined whether ectopic expression of A. thaliana ARK could rescue the moss ARK
mutant phenotypes. To this end, we attempted to express AtARK1, AtARK2, and
AtARK3 in moss ΔARKabc. For unknown reasons, transformants expressing AtARK1
could not be obtained, despite multiple attempts. However, lines stably expressing
AtARK2-mNG or AtARK3-mNG were successfully generated. The moss expressing
AtARK2 or AtARK3 grew slightly faster than the parental ΔARKabc line, and the colony
on the plate showed protenominal tissue more clearly (Fig. 7A, top). Robust growth of the
protomenal filament was also observed in the images of a 6-day-old protonema
regenerated from a single protoplast (Fig. 7A, bottom). Thus, AtARK2 and AtARK3
partially rescued this growth phenotype. In contrast, AtARK did not rescue the nuclear
position (Fig. 7B, arrowheads). The basal accumulation of chloroplasts in ΔARKabc
disappeared after ectopic expression of AtARK2 but not that of AtARK3 (Fig. 7B, C).
Interestingly, AtARK2 expression resulted in an apical shift of the chloroplast, which is
somewhat reminiscent of the KCBP knockout line [22]. Thus, we conclude that AtARK2
and AtARK3 can substitute for a subset of moss ARK functions.
Discussion

ARK is a versatile anterograde transporter in plants

A long sought-after in plant microtubule biology is an anterograde transporter that can carry a variety of cargos. Several data presented in this study suggest that it could be ARK, which is a widely conserved kinesin in plants.

First, the movement of three giant organelles and RabA2-marked vesicles was suppressed in the ARKabc triple mutant. Because movement towards the apex was suppressed and microtubule polarity was unchanged in the mutant, we concluded that ARK is responsible for the plus-end-directed motility of these organelles. Using ΔARKabcd, we also demonstrated that microtubule polymerisation dynamics were unperturbed in the absence of ARK. Thus, an alternative microtubule-based mechanism, namely, microtubule polymerisation-based pushing, is unlikely to be the cause of abnormal motility. The most natural interpretation is that ARK transports cargo to the microtubule plus-end. We speculate that the residual apical motility observed in the ΔARKabc mutant is driven by the intact ARKd protein. However, the possibility that additional motor families also play a role in the transport of these cargo cannot be ruled out. For example, in Arabidopsis, vesicular transport on cortical microtubules requires the FRA1 protein, which is a processive, plus-end-directed kinesin belonging to the kinesin-4 family.

Second, we detected the processive motility of ARKb proteins in vivo. The velocity and run length were comparable to those of retrograde motors (KCBP and KCH), supporting the idea that the tug-of-war between ARK and the retrograde motors underlies bidirectional transport of cargos. However, it remains unclear whether the motile ARKb-mNG signals represent cargo-carrying or cargo-free ARKb proteins. Because a significant population of ARK proteins are bound to microtubules but are not motile, an intriguing possibility is that cargo binding activates the motor and initiates motility. In contrast to wild-type ARKb, the rigor mutant did not show motility or rescue the mutant phenotype, which is consistent with the notion that ARKb acts as a transporter.

Third, the ARM domain in the tail region is critical for nuclear and chloroplast motility. The tail domain is essential for the function of cargo-carrying kinesins, because it is generally the binding site for cargo. In contrast, the tail of kinesin-13 or AtARK1 is dispensable for the regulation of microtubule dynamics. The criticality of ARM is consistent with the model in that it serves as the cargo-binding site.

Fourth, the mutant phenotype is pleiotropic. Our cellular analysis was conducted only in protoneural tissues, which are excellent for analysing microtubule-based transport. However, ARK plays an important role in rhizoids and gametophores, and their development is abnormal in ΔARKabcd. Determining which specific cellular defect is attributable to the phenotype is an interesting future research topic.

Finally, AtARK2 and AtARK3 partially restored organelle position and protoneural growth in the ΔARKabc mutant of P. patens. Thus, the transport function of ARK may be conserved in angiosperms. The AtARK2 mutant showed helical root growth, indicating that ARK2 is required for the proper growth of root epidermal cells. In contrast, ARK3 is thought to be essential for plant development. Abnormal distribution of stomata was observed upon cell type- and cell cycle-specific ARK3 knockdown in stomatal meristemoid mother cells. These phenotypes might be the consequence of...
mislocalisation of AtARK cargos. Whether AtARK1 has a similar function remains unclear. Given the phylogenetic distance and the reported cellular activity that has not been observed in moss, it might have differentiated to acquire novel functions. In the accompanying paper, liverwort ARK was shown to be required for organelle movement, further illustrating the evolutionary conservation of ARK as a transporter (Kanda et al. co-submitted).

Taken together, ARK is the strongest candidate for a universal anterograde transporter in plants, which carries a variety of cargos, including nuclei, chloroplasts, mitochondria, and vesicles, towards the microtubule plus ends (Fig. 7D).

**Microtubule-dependent transport supports tip growth**

Tip growth is a growth form found in several plant cell types, such as pollen tubes, root hairs, and moss protonemata, as well as in many other systems, including macroalgae, fungi, and neural axons in animals. Regulated plasticisation of the cell wall and vesicular transport of membrane and cell wall materials are important factors in the tip growth of walled organisms. Regarding the cytoskeleton, the primary player in tip growth is actin. For example, in *P. patens* protonemata, the addition of actin inhibitors immediately and completely stops tip growth. In contrast, microtubules are thought to play a role in determining growth orientation. Cell tip expansion and abnormal outgrowth in non-apical regions have been reported after the addition of microtubule-depolymerising drugs. However, the detailed mechanism by which microtubules define growth direction remains elusive.

Our analyses suggest that the microtubule- and ARK-dependent transport of actin regulatory molecules is critical for polarised tip growth. First, the rigor mutant of ARK could not restore tip growth. Second, actin regulatory molecules (RopGEF3, RopGEF6, and formin) and F-actin itself were less enriched at the apex in the ARK mutant. Third, and most notably, the growth phenotype was partially rescued by the artificial delivery of RopGEF3 to the apex by tailless ARKb. This result suggests that in wild-type moss, RopGEF or the upstream factor required for RopGEF localisation is transported by ARK. However, only a partial rescue of the phenotype by RopGEF force-localisation suggests that ARK transports other molecules critical for tip growth in addition to actin regulators. Identification of the cargo molecules would further clarify the versatility of ARK as a transporter and the detailed molecular mechanism of ARK-dependent tip growth.

Conceptually, the mechanism by which ARK-dependent anterograde transport regulates polarised cell growth is remarkably similar to what has been revealed in the fission yeast *Schizosaccharomyces pombe*, an organism with one of the best-studied cell growth systems. In fission yeast, bipolar cell growth during interphase is ensured by bipolar microtubule networks and the plus-end and cortical accumulation of the Tea1-Tea4 complex, which recruits formin For3. The molecule important for Tea1-Tea4 localisation is the processive, plus-end-directed kinesin Tea2. In the absence of Tea2, cells that initiate growth from improper sites are observed, which is likely driven by actin that is not restricted to a single site. An analogous mechanism is found in the filamentous fungus *Aspergillus nidulans*. Interestingly, the transport of polarity factors by anterograde motors is critical for cell morphogenesis also in mammalian cells. In epithelial cells, microtubules form uniform arrays along the apico-basolateral polarity axis, and several anterograde kinesins have been suggested to transport key polarity factors.
proteins associated with epithelial morphogenesis. Thus, long-distance transport of polarisation factors along microtubules might be a general scheme for polarised growth in a wide range of eukaryotic cells.

Materials and methods

P. patens culture and transformation

All strains in this study were derived from the Gransden ecotype of *Physcomitrium (Physcomitrella) patens*. *P. patens* culture and transformation protocols followed were as described by Yamada et al. (2016). Briefly, mosses were regularly cultured on BCDAT plates at 25 °C under continuous light illumination. A standard polyethylene glycol (PEG)-mediated method was exploited for transformation. Prior to transformation, sonicated protonemata were cultured on BCDAT agar medium for 5–6 days. Transgenic lines were selected using corresponding antibiotics. Line confirmation was conducted through genotyping PCR and visual inspection. Sequencing was performed to confirm the CRISPR mutant lines. The lines generated in this study are listed in Table S2.

Plasmid construction

The plasmids and primers used in this study are listed in Tables S3 and S4, respectively. CRISPR targets with high specificity were manually selected around the ATPase motif (P-loop) or microtubule binding site (called the Switch 2 region) in the motor domain of *ARK* genes. All target sequences were synthesised and ligated into the *BsaI* site of pPY156, which is based on pCasGuide/pUC18 and contains a hygromycin-resistant cassette. For endogenous tagging via homologous recombination, the plasmid was constructed using the In-Fusion HD Cloning Kit (Takara); 1–2 kb sequences of the 5' and 3' ends of the genes of interest flanked the fragment that consisted of an in-frame linker, mNeonGreen (mNG) coding sequence, Flag tag, and G418 resistant cassette. The mNG codon was optimised for expression in *Arabidopsis*. To generate the Rop4-mNG plasmid, the fusion constructs were constructed as described by Cheng et al. (2020). For the rescue experiment, the *ARKb* coding sequence was amplified from the moss cDNA library and ligated into the pENTR/D-TOPO vector containing the in-frame linker, mNG-coding sequence, and Flag tag, followed by the Gateway LR reaction (Invitrogen) into the pPY138 vector containing the *EF1a* promoter, G418 resistance cassette, and 1-kb sequences homologous to the *hb7* locus. Likewise, the gene sequences of *A. thaliana* *ARK2* and *ARK3* were amplified from a cDNA library (a gift from Dr Hidefumi Shinohara [Fukui Prefectural University]) and cloned into an overexpression vector containing the *EF1a* promoter.

Moss colony assay

To prepare the protoplasts, 5–7-day-old sonicated protonemata were digested with an 8% (w/v) mannitol solution supplemented with 1% (w/v) driselase for 0.5–1 h. After removing the driselase by washing twice with 8% mannitol solution, the protoplasts were resuspended in the protoplast regeneration liquid. After 4 h of incubation in the dark, the protoplasts were collected by centrifugation, resuspended in 7.5 mL of protoplast...
regeneration media (PRM) solution \(^7\), and spread onto three PRM plates covered with cellophane. The protoplasts were cultured for 3 days and transferred to a BCDAT plate. Images of 7-day-old moss protonemata were taken using a stereomicroscope SMZ800N (Nikon) equipped with an ILCE-QX1 camera (SONY). Nine-day-old protonemata were inoculated onto BCDAT plates and cultured for 3–5 weeks. Images of overall colonies or gametophores were captured using a C-765 Ultra Zoom digital camera (Olympus) or SMZ800N, respectively.

**Microscopy**

Time-lapse microscopy was performed as described by Nakaoka et al. (2012). Briefly, in the long-term time-lapse imaging experiments for the observation of protonemal cells, the protonemata were cultured on thin layers of BCD agarose in 6-well glass-bottom dishes for 5–7 days. Epifluorescence images were acquired with a Nikon Ti microscope (10× 0.45 NA lens, Zyla 4.2P CMOS camera (Andor), Nikon Intensilight Epi-fluorescence Illuminator) at intervals of 3–30 min with white light between acquisitions. For high-resolution imaging, protonemata were inoculated onto the agar pad in a 35 mm glass-bottom dish, followed by culturing for 5–7 days. Confocal imaging was performed with a Nikon Ti microscope attached to a CSU-X1 spinning disk confocal scanner unit (Yokogawa), EMCCD camera (ImagEM, Hamamatsu), and three laser lines (637, 561, and 488 nm). Lenses were selected depending on the experiment (40× 1.30 NA, 60× 1.40 NA, and 100× 1.45 NA). Oblique illumination fluorescence microscopy was performed using a Nikon Ti microscope attached to a total internal reflection fluorescence (TIRF) unit, 100× 1.49-NA lens, GEMINI split view (Hamamatsu), and EMCCD camera Evolve (Roper) \(^8\). The samples for oblique imaging were prepared with a microfluidic device of 15 μm height. Stock solutions of oryzalin, latrunculin A, and FM4-64 in DMSO were diluted with distilled water to working concentrations of 10 μM oryzalin, 25 μM latrunculin A, and 10 μM FM4-64. Prior to drug addition, the protonemal tissue on the agarose pad was preincubated in water for 1 h for absorption. After water removal, 1 mL of the drug solution was added, and image acquisition was started immediately. DMSO was used as a control. To induce RNAi, 1 μM β-estradiol was added to the 4–5-day-old protonemata on sample dishes 12 h prior to observation. Most of the imaging was performed at 22–25 °C in the dark, except for the images shown in Figure S5A, which were taken under continuous light.

**Image data analysis**

All raw data processing and measurements were performed using the Fiji software.

*Moss colony size.* The moss colony images were outlined automatically, and the area was measured using Fiji.

*Velocity and run length of ARKb.* Oblique illumination time-lapse images of the endoplasmic microtubules in protonemal cells were taken every 200 ms. The microtubules on which single or multiple ARKb-mNeonGreen particles were continuously moving were manually selected and analysed. A kymograph was generated.
along the microtubule, and the inclination and distance of the line derived from ARKb particles were measured as the velocity and run length, respectively.

**Microtubule plus-end dynamics and orientation.** To quantify the plus end growth/shrinkage rate and rescue/catastrophe frequency, we followed the protocol described by Leong et al. (2020). Briefly, oblique illumination time-lapse images of endoplasmic microtubules in protonemal cells were taken every 3 s for 3 min. Then, a 5 × 6 µm area was randomly selected in each cell, and kymographs of every traceable microtubule plus end in the area were created. The inclination of the microtubules in the kymographs, which corresponded to the growth or shrinkage rate, was measured. To determine catastrophe or rescue frequency, the number of events was counted and divided by the observed growth or shrinkage duration of the microtubule, respectively. The growth rate at the cell apex was specifically determined using EB1-Citrine imaging, which tracks growing plus ends. Time-lapse images of EB1-Citrine were acquired every 3 s using a spinning-disc confocal microscope. Kymographs were generated along the edge of the caulonemal cell apex. The inclination of EB1-Citrine signals was measured to determine the microtubule growth rate. To analyse microtubule orientation in interphase apical cells, cells were observed for > 150 min after anaphase onset. Kymographs were generated along the edges of the cells, and the directionality of EB1-Citrine comets was measured to determine the overall orientation of the microtubules.

**Actin growth rate.** Time-lapse images of apical cell tips of the Lifeact-mNG expressing line were taken every 200 ms using TIRF microscopy. The elongating Lifeact-mNG at the cell tip was tracked and used to generate kymographs. The inclination of the Lifeact-mNG on the kymograph was measured as the growth rate of the actin filament. We noted that actin dynamics parameters were sensitive to the expression levels of Lifeact-mNG. Therefore, we used the same parental line expressing Lifeact-mNG in this experiment and analysis.

**Spindle position and cell length.** For apical cells, time-lapse images were obtained every 3 min, and the distance between the basal cell wall and cell tip (i.e. cell length) or the spindle equator was measured just before anaphase onset. The relative position of the metaphase spindle was determined using division. The length of the subapical cells was measured based on a snapshot of caulonemal filaments.

**Subapical cell length.** Images of moss protonemata stained with FM4-64 were obtained. The lengths of the subapical cells of the caulonemal filaments were measured.

**Cell cycle duration.** Time-lapse images of the caulonemal cells were obtained every 10 min. The duration between the nuclear envelope breakdown (NEBD) of the mother cell and the NEBD of the daughter apical cell was measured.

**Nuclear movement.** Time-lapse images were taken every 3 min. The distance between the cell plate and centre of the nucleus was measured in apical cells before and after cell
division. Similarly, the distance between the apical cell wall and the centre of the nucleus was measured in subapical cells with a bulge.

**Chloroplast distribution.** Time-lapse images were taken every 3 min. The intensity of chloroplast autofluorescence along the long axis of caulonemal apical cells was measured 150 min after anaphase onset, and the background intensity of each image was subtracted. The intensity of each pixel on the drawn line was divided by the mean intensity of the entire length of the line to get relative intensity. The cells were divided into ten sections, and the average relative intensity of each section is displayed.

**Mitochondrial movement.** To visualise mitochondria, γ-F1ATPase-mNG was expressed under the EF1α promoter. A kymograph was created from images acquired every 2 s using oblique illumination fluorescence microscopy. To quantify mitochondrial relocation, time-lapse images of the apical side of caulonemal apical cells were obtained every 10 s for 6 min using spinning-disc confocal microscopy. The majority of the microtubules in this area are oriented in such a manner that the plus ends face the apex, enabling a reliable estimate of whether mitochondria tend to move towards the plus or minus end. In this analysis, 15 μm² was selected in the area between the cell apex and nucleus, and six mitochondria that were clearly identified were analysed. The position of each mitochondrion in the first and last frame was compared; based on the overall microtubule polarity, translocation towards the tip was interpreted as anterograde motility.

**Intensity of mNG-RabA2b and For2A-mNG.** The apex of caulonemal cells in which mNG-RabA2b or For2A-mNG was expressed was imaged using a z-series taken every 0.5 μm for a range of 15 μm. The sums of the slices were made by z-projection using the Fiji software. The mean intensity within approximately 2 μm² of the punctate signals was measured and subtracted by the background intensity.

**Movement of mNG-RabA2b puncta.** Images of caulonemal apical cells expressing mNG-RabA2b were taken every 200 ms using a spinning-disc confocal microscope. The number of signal puncta moving >10 μm in a minute was counted for each cell.

**Tip growth rate.** Time-lapse epifluorescent images of protonemata were obtained every 3 min using a 10× lens. Kymographs were created along the axes of growing caulonemal filaments, and the slope of the kymographs was measured to obtain the growth rate.

**Cell morphology observation under ARKd RNAi.** The moss protonemata were cultured on BCDAT for 4–5 days, and 1 μM β-estradiol or the control (0.1% DMSO) was added to the plant. After four days of incubation following drug addition, the morphology of the apical cells was observed and counted.
Frequency of actin foci formation at the cell apex. The apexes of the cells expressing Lifeact-mNG was imaged using a z-series taken every 0.5 µm for a range of 8 µm for 10 min at 30 s intervals. Following the method described by Yamada and Goshima, (2018), the z-stack images were processed by maximum z-projection with Fiji, and the number of time frames in which the actin focus was clearly observed was counted. The ratio of time frames with clear foci was used to determine the frequency of foci formation.

Intensity of Rop4-mNG and RopGEFs-mNG. The protonemal cell apex of the cell line expressing Rop4-mNG or RopGEFs-mNG was imaged using a z-series taken every 0.5 µm for a range of 15 µm. A slice showing the centre of the cell was selected. The total intensity of mNG signals was measured along the cell edge by drawing a line (width = 0.4 µm) (subtracted by the cytoplasmic background intensity). ROP-GEF6 was distributed more broadly than ROP-GEF3. For the analysis of the temporal change of the intensity under the drug treatment, time-lapse images were acquired every 1 or 3 min using a z-series taken every 1.5 µm for a range of 6 µm. The images were quantified in an identical manner.

Statistical analysis

Welch’s two-tailed t-test was used when the samples to be compared comprised two groups. Tukey’s multiple comparison test was used to analyse datasets that included more than two groups. All statistical analyses were performed using the R software. The data distribution was assumed to be normal, but this was not formally tested. Obtained P values are denoted as follows: *, P < 0.05; **, P < 0.01; ***, P < 0.001; and ****, P < 0.0001. Data from multiple experiments were combined because of insufficient sample numbers in a single experiment, unless otherwise stated.

Accession numbers

The gene sequences used in this study are available in Phytozome under the following accession numbers: AtARK1 (AT3G54870.3), AtARK2 (AT1G01950.3), AtARK3 (AT1G12430.1), PpARKa (Pp3c27_850V3.1), PpARKb (Pp3c16_2830V3.1), PpARKc (Pp3c6_19520V3.1), PpARKd (Pp3c2_28160V3.1), and MpARK (Mapoly0114s0009.1).

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**Figure legends**

(A) Phylogenetic analysis of *ARK*: moss *Physcomitrium patens* (Pp), Brassica *Arabidopsis thaliana* (At), and liverwort *Marchantia polymorpha* (Mp). Amino acid sequences were collected from the database (accession numbers are listed in the Methods section), aligned with MAFFT, and gaps were deleted. The phylogenetic tree was constructed using the neighbour-joining method and MEGAX software, and its reliability was assessed using 1,000 bootstrapping trials. The bar indicates 0.05 amino acid substitutions per site.

(B) Representative images of 5-week-old moss colonies regenerated from a single protoplast (top row), 6-day-old protonemata (second row), gametophores and rhizoids.

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(third row, where rhizoids are marked with red arrowheads), and the leaf surface (bottom row). The mosses used in this experiment were control (mCherry-α tubulin), ΔARKabc, ΔARKabcd, and overexpression/rescue lines (ΔARKabc/ARKb-mNG OX and ΔARKabc/ARKb (T169N) -mNG OX). Scale bars: 1 mm (top row), 200 μm (second row), 1 mm (third row), and 100 μm (bottom).

(C) Colony area comparison. Colonies were cultured from a single protoplast for three weeks on BCDAT medium. The same moss lines as those in (B) were used in this experiment. The mean area (mm²) was 41.0 ± 2.28 (control, ±SEM, n = 30), 4.91 ± 0.267 (ΔARKabc, ±SEM, n = 45), 30.7 ± 2.42 (ΔARKabc/ARKb-mNG OX, ±SEM, n = 18), 4.26 ± 0.501 (ΔARKabc/ARKb (T169N) -mNG OX, ±SEM, n = 18), 1.90 ± 0.188 (ARKabcd, ±SEM, n = 49). P-values were calculated using Tukey’s multiple comparison test; P < 0.0000001 (control - ΔARKabc), P < 0.0000001 (ΔARKabc - ΔARKabc/ARKb-mNG OX).

**Fig. 2 ARK transports multiple organelles in protoplasmic cells**

(A) Movement of the nuclei and chloroplasts in protoplasmic apical cells. The onset time of anaphase was set to 0 min. The yellow circles indicate the positions of the nuclei. Yellow boxes are highlighted in (D). Images were acquired with z-stacks and processed using maximum z-projection: 3 μm × 3 (control mCherry-α tubulin line) or 2.5 μm × 3 sections (ΔARKabc and ΔARKabc/ARKb-mNG OX). Bar, 20 μm.

(B) Nuclear movement before and after apical cell division. The temporal change in the distance between the nucleus and the cell plate (set at position 0) was plotted with SEM. n = 9 cells (control), n = 10 cells (ΔARKabc), n = 10 cells (ΔARKabc/ARKb-mNG OX).

(C) Comparison of the relative position of the metaphase plate of the spindle in dividing apical cells. The relative position was determined by dividing the distance between the metaphase plate and the basal cell wall by that between the cell tip and the basal cell wall. mCherry-α tubulin was used as the control. The mean relative position was 62.2 ± 0.930% (control, ±SEM, n = 10), 41.7 ± 1.71% (ΔARKabc, ±SEM, n = 10), 58.3 ± 0.880% (ARKabc/ARKb-mNG OX, ±SEM, n = 11), 42.1 ± 1.67% (ΔARKabc/ARKb (T169N) -mNG OX, ±SEM, n = 5). P-values were calculated using Tukey’s multiple comparison test; P < 0.0000001 (control - ΔARKabc), P < 0.0000001 (ΔARKabc - ΔARKabc/ARKb-mNG OX).

(D) Movement of individual chloroplasts 88–96 min after anaphase onset. Yellow boxes in (A) are highlighted. Scale bar, 5 μm.

(E) Relative intensity of chloroplasts in apical cells 150 min after anaphase onset. Chloroplast accumulation near the basal cell wall was observed in ΔARKabc and ΔARKabc/ARKb (T169N) -mNG OX plants. mCherry-α tubulin was used as the control. The quantification methods are described in the Methods section.

(F) Bidirectional motility of mitochondria on a microtubule in the control line (mCherry-α Tubulin/γ-F1ATPase-mNG). The right panel shows the kymograph. Scale bars: 1 μm (horizontal) and 30 s (vertical). The growing microtubule end (i.e. the plus end) is indicated by an arrow.

(G) Frequency of tip-directed motility of mitochondria in interphase apical cells. The quantification method has been described in the Methods section. The mean frequency was 90.3 ± 4.33% (control, ± SEM, n = 12 cells), 52.8 ± 8.42% (ΔARKabc,
± SEM, n = 12 cells). P-values were calculated using Welch’s two-sample t-test; P = 0.001067.

Fig. 3 ARK promotes the movement of RabA2b-marked secretory vesicles

(A) and (B) Accumulation of mNG-RabA2b at the cell tip was reduced in ΔARKabc. mCherry-α-tubulin/RabA2b-mNG was used as control. Scale bar, 5 µm. The mean intensity in (B) was 1.22 ± 0.0845 (control, ±SEM, n = 15), 0.69 ± 0.0507 (ΔARKabc, ±SEM, n = 12), and 0.970 ± 0.0645 (ΔARKabc/ARKb-mNG OX, ±SEM, n = 7). P-values were calculated using Tukey’s multiple comparison test; P = 0.0000215 (control - ΔARKabc), P = 0.0704068 (ΔARKabc - ΔARKabc/ARKb-mNG OX).

(C) Representative images of RabA2b-positive vesicles (arrowheads) and number of motile RabA2b-positive vesicles in the absence of microtubules or actin. Scale bar, 5 µm. The number of mNG puncta moving >10 µm in a minute was counted in each cell. The mean number was 2.1 ± 0.46 (control, ±SEM, n = 10 cells), 0.0± 0.0 (10 µM oryzalin, ±SEM, n = 5 cells), 0.3 ± 0.20 (25 µM latrunculin A, ±SEM, n = 10 cells), 0.0± 0.0 (10 µM oryzalin + 25 µM latrunculin A, ±SEM, n = 10 cells).

(D) Number of RabA2b-positive vesicles moving for >10 µm in a minute. mCherry-α-tubulin/RabA2b-mNG was used as control. The mean number was 2.3 ± 0.30 (control, ±SEM, n = 20 cells), 1.1 ± 0.31 (ΔARKabc, ±SEM, n = 17 cells), 2.8 ± 0.60 (ΔARKabc/ARKb-mNG OX, ±SEM, n = 17 cells). P-values were calculated using Tukey’s multiple comparison test; P = 0.1998938 (control - ΔARKabc), P = 0.0144199 (ΔARKabc - ΔARKabc/ARKb-mNG OX).

Fig. 4 ARKb-mNG shows processive motility in vivo

(A) Localisation of ARKb-mNG on endoplasmic microtubules in protonemal cells. Giant green signals near the scale bar represent autofluorescent chloroplasts. Scale bar, 5 µm.

(B) Processive movement of ARKb-mNG along a microtubule. Arrowheads indicate ARKb-mNG signals. Scale bar, 500 nm.

(C) Kymographs of ARKb-mNG (left) and ARKb (T169N)-mNG (right) on microtubules. Scale bar: 5 µm (horizontal) and 5 s (vertical).

(D) Velocity and run length of moving ARKb-mNG signals. Mean values were 572 ± 243 nm/s (velocity, mean ±SD, n = 115) and 986 ± 416 nm (run length, mean ±SD, n = 106).

Fig. 5 Cell tip growth was severely suppressed in the absence of ARK

(A) Representative images of protonemal cells. The plasma membrane was visualised using FM4-64. The mCherry-α-tubulin line was used as the control. ΔARKabcd exhibited additional nuclear signals derived from the mCherry-tagged histone. Scale bar, 100 µm.

(B) Comparison of subapical cell length. The mean length was 122 ± 4.83 µm (control, ±SEM, n = 12), 70.2 ± 2.67 µm (ΔARKabc, ±SEM, n = 13), 137 ± 3.97 µm (ΔARKabc/ARKb-mNG OX, ±SEM, n = 13). P-values were calculated using Tukey’s multiple comparison test; P < 0.0000001 (control - ΔARKabc), P < 0.0000001 (ΔARKabc - ΔARKabc/ARKb-mNG OX).

(C) Comparison of tip growth rates of caulonemal filament. The mCherry-α-tubulin line was used as the control. The mean rate was 20.8 ± 0.962 µm/h (control, ±SEM, n =
30, 10.5 ± 0.455 µm/h (ΔARKabc, ±SEM, n = 29), 22.1 ± 1.10 µm/h (ΔARKabc/ARKb-mNG OX, ±SEM, n = 18), 8.23 ± 0.56 µm/h (ΔARKabc/ARKb-T169N) -mNG OX, ±SEM, n = 13). P-values were calculated by Tukey’s multiple comparison test; P < 0.000001 (control - ΔARKabc), P < 0.0000011 (ΔARKabc - ΔARKabc/ARKb-mNG OX).

(D) Representative images of actin foci at the cell tip. Scale bar, 5 µm.

(E) Frequency of actin focusing. The mean frequencies were 68.8 ± 5.84% (ΔARKabc, ±SEM, n = 9), 83.6 ± 5.56% (ΔARKabc / ARKB-mNG OX, ±SEM, n = 9). P-values were calculated using Welch’s two-sample t-test: P = 0.08485 (ΔARKabc - ΔARKabc/ARKb-mNG OX).

(F) Comparison of actin growth rates near the cell tip. F-actin was visualised using Lifeact-mNG. The mean rate was 1.79 ± 0.0790 µm/sec (ΔARKabc, ±SEM, n = 14), 2.07 ± 0.084 µm/sec (ΔARKabc/ARKb-mNG OX, ±SEM, n = 23). The P-value was calculated using Welch’s two-sample t-test: P = 0.02227 (ΔARKabc - ΔARKabc/ARKb-mNG OX).

Fig. 6 ARK and microtubules are necessary for the tip localisation of RopGEFs

(A) and (B) RopGEF3 and RopGEF6 levels at the cell tip were decreased in the ARK mutant. (A) Representative images of RopGEF3-mNG and RopGEF6-mNG at the cell tip. Scale bar, 5 µm. (B) Comparison of the intensity of RopGEF3-mNG and RopGEF6-mNG at the cell apex. The mean intensity of RopGEF3-mNG was 1.82 ± 0.202 (control, ±SEM, n = 6), 1.20 ± 0.0792 (ΔARKabc, ±SEM, n = 7). P-values were calculated using Welch’s two-sample t-test: P = 0.0119944 (control - ΔARKabc). The mean intensity of RopGEF6-mNG was 1.85 ± 0.0905 (control, ±SEM, n = 7), 1.23 ± 0.0825 (ΔARKabc, ±SEM, n = 10). P-values were calculated using Welch’s two-sample t-test: P = 0.0003975 (control - ΔARKabc), P = 0.0000050 (ΔARKabc - ΔARKabc/ARKb-mNG OX).

(C) and (D) Representative images and comparison of For2A-mNG intensity at the cell tip. The mean intensity was 1.03 ± 0.0978 (control, ±SEM, n = 10) and 0.802 ± 0.0647 (ΔARKabc, ±SEM, n = 11). P-value was calculated using Welch’s two-sample t-test: P = 0.06873 (control - ΔARKabc).

(E) Motor-RopGEF-fusion constructs used in this study. Tailless ARKb and mNeonGreen-FLAG were fused to the N-terminal and C-terminal sides of RopGEF3, respectively. Scale bar: 100 amino acids.

(F) Representative images of ΔARKabc moss expressing native or motor-fused RopGEF3 (ARKb(ΔTail)-RopGEF3-mNG). Top row: 5-week-old moss colonies grown from a single protoplast; second row: 6-day-old protonemata; third row: tip localisation of the fusion protein. Scale bars: 2 mm (top row), 200 µm (second row), and 5 µm (third row).

(G) Relative intensity of chloroplasts along the apical cell at 150 min after anaphase onset. Chloroplast accumulation near the basal cell wall observed in ΔARKabc was not rescued by the fusion RopGEF3 construct.

(H) Area comparison of 5-week-old moss colonies. Colonies were cultured on BCDAT medium from a single protoplast for five weeks. The mean area (mm²) was 28.2 ± 2.42 (ΔARKabc, ±SEM, n = 18), 50.5 ± 3.08 (ΔARKabc/RopGEF3-mNG OX, ±SEM, n = 18), 91.5 ± 4.41 (ΔARKabc/ARKb(Δtail)-RopGEF3-mNG, ±SEM, n = 18).
values were calculated using Tukey’s multiple comparison test; P = 0.1878188

\( \Delta ARK_{abc} - \Delta ARK_{abc}/\text{RopGEF3-mNG OX}, P < 0.0000001 (\Delta ARK_{abc} - \Delta ARK_{abc}/\text{ARKb-RopGEF3-mNG}) \).

Fig. 7 AtARK2 and AtARK3 expression partially rescues moss AtARKbc

(A) Representative images of 5-week-old moss colonies regenerated from a single protoplast (top) and 6-day-old protonemata (bottom). Scale bars: 2 mm (top) and 200 \( \mu m \) (bottom).

(B) Representative images of protonemal apical cells. Arrowheads and white lines indicate the positions of the nucleus and the cell plate, respectively. Scale bar, 100 \( \mu m \).

(C) Relative intensity of chloroplasts along the apical cell at 150 min after anaphase onset (+SEM). The basal accumulation of chloroplasts in AtARKbc was abolished after the ectopic expression of AtARK2 but not of AtARK3.

(D) Model for microtubule-dependent transport in Physcomitrium patens protonemal cells. Giant organelles, such as nuclei and chloroplasts, are transported to the plus and minus ends of the microtubules by ARK and kinesin-14s (KCH and KCBP), respectively (i.e. tug-of-war). ARK also transports secretory vesicles, which are presumably passed to myosin XI at the cell tip for exocytosis. The proteins and cell wall materials required for tip growth are also candidate cargos for ARK, as ARK depletion severely suppresses polarised cell growth.

Supplemental Fig. 1 Establishment of Physcomitrium patens ARK mutants

(A) Representative images of 3-week-old moss colonies of ARK mutants. mCherry-\( \alpha \)-tubulin was used as a control. Scale bar, 5 mm.

(B) Sequencing revealed frameshift mutations in the \( \Delta ARK_{abc} \) and \( \Delta ARK_{abcd} \) sequences used in this study (displayed as SnapGene sequence files).

Supplemental Fig. 2 ARK deletion does not affect microtubule dynamics

(A) Comparison of microtubule dynamics. Upper row, control (mCherry-\( \alpha \)-tubulin) and \( \Delta ARK_{abc} \); lower row, control (GFP-\( \alpha \)-tubulin/Histone-mCherry), and \( \Delta ARK_{abcd} \). The mean ± SEM, number of samples, and P-values are shown in Table S1.

(B) Comparison of microtubule orientation in apical cells based on EB1-Citrine tracking. The quantification method is described in the Methods section. The frequency of tip-directed movement (%) was 97.0 ± 1.16 (mCherry-\( \alpha \)-Tubulin, ±SEM, n = 18) and 88.5 ± 2.61 (\( \Delta ARK_{abc} \), ±SEM, n = 8). The P-value was calculated using Welch’s two-sample t-test; P = 0.007365.

Supplemental Fig. 3 The truncation-rescue assay reveals the essentiality of ARM repeats

(A) Truncated ARKb-mNG constructs used in this experiment. The protein domains were predicted using NCBI’s conserved domain database (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and SMART (http://smart.embl.de).

(B) Area comparison of 3-week-old moss colonies. Colonies were cultured from a single protoplast for three weeks on BCDAT medium. The datasets of \( \Delta ARK_{abc}/\text{FL} \) (full-
length) are identical to those shown in Fig. 1C. The mean area (mm²) was 4.88 ± 0.651 (ΔARKabc/ΔARM, ±SEM, n = 18), 7.04 ± 1.07 (ΔARKabc/ΔTail, ±SEM, n = 18).

(C) Relative intensity of chloroplasts along the apical cell at 150 min after anaphase onset. The chloroplasts remained accumulated near the basal cell wall after the expression of the ΔARM or ΔTail construct.

Supplemental Fig. 4 ARK deletion affects cell length, but not the cell cycle duration or Rop4 localisation

(A) Comparison of cell cycle durations. The mean duration (h) was 7.43 ± 0.307 (control, ±SEM, n = 24), 7.58 ± 0.232 (ΔARKabc, ±SEM, n = 13), 6.93 ± 0.220 (ΔARKabc/ARKb-mNG OX, ±SEM, n = 36).

(B) Comparison of tip growth rates in the presence of 500 pM or 50 nM latrunculin A (LatA). The mCherry-α-tubulin line was used for drug treatment. The mean rate (µm/h) was 9.69 ± 1.20 (ΔARKabc, ±SEM, n = 5), 23.0 ± 1.00 (control [+0.5% DMSO], ±SEM, n = 5), 16.7 ± 1.20 (+500 pM LatA, ±SEM, n = 6), 9.64 ± 0.702 (+50 nM LatA, ±SEM, n = 17).

(C) and (D) Representative images and comparison of intensities of functional Rop4-mNG at the cell tip. The mean intensity was 1.29 ± 0.0724 (control, ±SEM, n = 6), 1.54 ± 0.140 (ΔARKabc, ±SEM, n = 11).

(E) The apical accumulation of RopGEF3 and RopGEF6 depends on the microtubules. Snapshots of the same cell before and 50-min after oryzalin addition are shown. DMSO was used as a control. mCherry-α-tubulin-expressing RopGEF3-mNG or RopGEF6-mNG was used in this experiment. Scale bar, 5 µm.

(F) Temporal changes in RopGEF3-mNG or RopGEF6-mNG intensity at the cell tip after drug addition. A decrease in localisation was observed after microtubule disruption with oryzalin. Intensities relative to those before drug treatment are plotted (±SEM, n = 6–13).

Supplemental Fig. 5 RNAi of ARKd in ΔARKabc causes abnormal outgrowth

(A) Representative image sequences of abnormal cell growth after ARKd RNAi induction by β-estradiol. Left, outgrowth in the apical cell; right, outgrowth in the subapical cell. Scale bars, 100 µm.

(B) Comparison of apical cell shape after 1 µM β-estradiol or control 0.1% DMSO treatment. The numbers shown in the bar graphs indicate the actual number of counted cells.

Supplemental movie legends

Movie 1 Overall basal motility of chloroplasts after cell division in ΔARKabc

Time-lapse movie of microtubules (mCherry-α-tubulin, magenta) and chloroplasts (autofluorescence, cyan) in apical cells after anaphase onset (0.0 min). The bright magenta signals on the left represent the anaphase spindles and phragmoplasts. White arrowheads indicate the positions of the nuclei. Control (expressing mCherry-α-tubulin) and ΔARKabc lines are also shown. Scale bar, 20 µm.
Movie 2 Motility of ARKb-mNG on cytoplasmic microtubules in vivo

Time-lapse movie of ARKb-mNG obtained using oblique illumination fluorescence microscopy. White arrowheads indicate the puncta of ARKb-mNG that move processively and unidirectionally on the microtubules. Magenta, tubulin; green, ARKb-mNG. Scale bar, 5 µm.
Fig. 1 ARK disruption causes severe growth defects

(A) Phylogenetic analysis of ARK: moss Physcomitrium patens (Pp), Brassica Arabidopsis thaliana (At), and liverwort Marchantia polymorpha (Mp). Amino acid sequences were collected from the database (accession numbers are listed in the Methods section), aligned with MAFFT, and gaps were deleted. The phylogenetic tree was constructed using the neighbour-joining method and MEGAX software, and its reliability was assessed using 1,000 bootstrapping trials. The bar indicates 0.05 amino acid substitutions per site.

(B) Representative images of 5-week-old moss colonies regenerated from a single protoplast (top row), 6-day-old protonemata (second row), gametophores and rhizoids (third row, where rhizoids are marked with red arrowheads), and the leaf surface (bottom row). The mosses used in this experiment were control (mCherry-α-tubulin), ΔARKabc, ΔARKabcd, and overexpression/rescue lines (ΔARKabc/ARKb-mNG OX and ΔARKabcd/ARKb (T169N) -mNG OX). Scale bars: 1 mm (top row), 200 µm (second row), 1 mm (third row), and 100 µm (bottom).

(C) Colony area comparison. Colonies were cultured from a single protoplast for three weeks on BCDAT medium. The same moss lines as those in (B) were used in this experiment. The mean area (mm²) was 41.0 ± 2.28 (control, ±SEM, n = 30), 4.91 ± 0.267 (ΔARKabc, ±SEM, n = 45), 30.7 ± 2.42 (ΔARKabcd/ARKb-mNG OX, ±SEM, n = 18), 4.26 ± 0.501 (ΔARKabc/ARKb (T169N) -mNG OX, ±SEM, n = 18), 1.90 ± 0.188 (ΔARKabcd, ±SEM, n = 49). P-values were calculated using Tukey's multiple comparison test; P < 0.0000001 (control - ΔARKabc), P < 0.0000001 (ΔARKabc - ΔARKabcd/ARKb-mNG OX).
Fig. 2 ARK transports multiple organelles in protonemal cells
(A) Movement of the nuclei and chloroplasts in protonemal apical cells. The onset time of anaphase was set to 0 min. The yellow circles indicate the positions of the nuclei. Yellow boxes are highlighted in (D). Images were acquired with z-stacks and processed using maximum z-projection: 3 µm × 3 (control mCherry-α-Tubulin line) or 2.5 µm × 3 sections (ΔARKabc and ΔARKabc/ARKb-mNG OX). Bar, 20 µm.
(B) Nuclear movement before and after apical cell division. The temporal change in the distance between the nucleus and the cell plate (set at position 0) was plotted with SEM. = 9 cells (control), ° = 10 cells (ΔARKabc), ° = 10 cells (ΔARKabc/ARKb-mNG OX).
(C) Comparison of the relative position of the metaphase plate of the spindle in dividing apical cells. The relative position was determined by dividing the distance between the metaphase plate and the basal cell wall by that between the cell tip and the basal cell wall. mCherry-α-tubulin was used as the control. The mean relative position was 62.2 ± 0.930K (control, ±SEM, ° = 10), 41.7 ± 1.711% (ΔARKabc, ±SEM, ° = 10), 58.3 ± 0.880% (ΔARKabc/ARKb-mNG OX, ±SEM, ° = 11). P-values were calculated using Tukey’s multiple comparison test; P = 0.0000001 (control - ΔARKabc), P = 0.0000001 (ΔARKabc - ΔARKabc/ARKb-mNG OX).
(D) Movement of individual chloroplasts 88–96 min after anaphase onset. Yellow boxes in (A) are highlighted. Scale bar, 5 µm.
(E) Relative intensity of chloroplasts in apical cells 150 min after anaphase onset. Chloroplast accumulation near the basal cell wall was observed in ΔARKabc and ΔARKabc/ARKb (T169N) -mNG OX plants. mCherry-α-tubulin was used as the control. The quantification methods are described in the Methods section.
(F) Bidirectional motility of mitochondria on a microtubule in the control line (mCherry-α-Tubulin/F1ATPase-mNG). The right panel shows the kymograph. Scale bars: 1 µm (horizontal) and 30 s (vertical). The growing microtubule end (i.e. the plus end) is indicated by an arrow.
(G) Frequency of tip-directed motility of mitochondria in interphase apical cells. The quantification method has been described in the Methods section. The mean frequency was 90.3 ± 4.33% (control, ± SEM, ° = 12 cells), 52.8 ± 8.42% (ΔARKabc, ± SEM, ° = 12 cells). P-values were calculated using Welch’s two-sample t-test; P = 0.00106.
Fig. 3 ARK promotes the movement of RabA2b-marked secretory vesicles

(A) and (B) Accumulation of mNG-RabA2b at the cell tip was reduced in ΔARKabc. mCherry-α-tubulin/RabA2b-mNG was used as control. Scale bar, 5 µm. The mean intensity in (B) was 1.22 ± 0.0845 (control, ±SEM, n = 15), 0.69 ± 0.0507 (ΔARKabc, ±SEM, n = 12), and 0.970 ± 0.0645 (ΔARKabc/ARKb-mNG OX, ±SEM, n = 7). P-values were calculated using Tukey’s multiple comparison test: P = 0.0000215 (control - ΔARKabc), P = 0.004068 (ΔARKabc - ΔARKabc/ARKb-mNG OX).

(C) Representative images of RabA2b-positive vesicles (arrowheads) and number of motile RabA2b-positive vesicles in the absence of microtubules or actin. Scale bar, 5 µm. The number of mNG puncta moving >10 µm in a minute was counted in each cell. The mean number was 2.1 ± 0.46 (control, ±SEM, n = 10 cells), 0.0 ± 0.0 (10 µM oryzalin, ±SEM, n = 5 cells), 0.3 ± 0.20 (25 µM latrunculin A, ±SEM, n = 10 cells), 0.0 ± 0.0 (10 µM oryzalin + 25 µM latrunculin A, ±SEM, n = 10 cells).

(D) Number of RabA2b-positive vesicles moving for >10 µm in a minute. mCherry-α-tubulin/RabA2b-mNG was used as control. The mean number was 2.3 ± 0.30 (control, ±SEM, n = 20 cells), 1.1 ± 0.31 (ΔARKabc, ±SEM, n = 17 cells), 2.8 ± 0.60 (ΔARKabc/ARKb-mNG OX, ±SEM, n = 17 cells). P-values were calculated using Tukey’s multiple comparison test: P = 0.1998938 (control - ΔARKabc), P = 0.0144199 (ΔARKabc - ΔARKabc/ARKb-mNG OX).
Fig. 4 ARKb-mNG shows processive motility in vivo

(A) Localization of ARKb-mNG on endoplasmic microtubules in protonemal cells. Giant green signals near the scale bar represent autofluorescent chloroplasts. Scale bar, 5 µm.

(B) Processive movement of ARKb-mNG along a microtubule. Arrowheads indicate ARKb-mNG signals. Scale bar, 500 nm.

(C) Kymographs of ARKb-mNG (left) and ARKb (T169N)-mNG (right) on microtubules. Scale bar: 5 µm (horizontal) and 5 s (vertical).

(D) Velocity and run length of moving ARKb-mNG signals. Mean values were 572 ± 243 nm/s (velocity, mean ± SD, n = 115) and 966 ± 416 nm (run length, mean ± SD, n = 106).
Fig. 5 Cell tip growth was severely suppressed in the absence of ARK

(A) Representative images of protonemal cells. The plasma membrane was visualised using FM4-64. The mCherry-α-tubulin line was used as the control. ΔARKabc exhibited additional nuclear signals derived from the mCherry-tagged histone. Scale bar, 100 µm.

(B) Comparison of subapical cell length. The mean length was 122 ± 4.83 µm (control, ±SEM, n = 12), 70.2 ± 2.67 µm (ΔARKabc, ±SEM, n = 13), 137 ± 3.97 µm (ΔARKabc/ARKb-mNG OX, ±SEM, n = 13). P-values were calculated using Tukey’s multiple comparison test; P < 0.0000001 (control - ΔARKabc), P < 0.0000001 (ΔARKabc - ΔARKabc/ARKb-mNG OX).

(C) Comparison of tip growth rates of caulonemal filament. The mCherry-α-tubulin line was used as the control. The mean rate was 20.8 ± 0.962 µm/h (control, ±SEM, n = 30), 10.5 ± 0.455 µm/h (ΔARKabc, ±SEM, n = 29), 22.1 ± 1.10 µm/h (ΔARKabc/ARKb-mNG OX, ±SEM, n = 18), 8.23 ± 0.56 µm/h (ΔARKabc/ARKb(T169N)-mNG OX, ±SEM, n = 13). P-values were calculated by Tukey’s multiple comparison test: P = 0.0000001 (ΔARKabc - ΔARKabc/ARKb-mNG OX).

(D) Representative images of actin foci at the cell tip. Scale bar, 5 µm.

(E) Frequency of actin focusing. The mean frequencies were 68.8 ± 5.84% (ΔARKabc, ±SEM, n = 9), 83.6 ± 5.56% (ΔARKabc/ARKb-mNG OX, ±SEM, n = 9). P-values were calculated using Welch’s two-sample t-test: P = 0.08465 (ΔARKabc - ΔARKabc/ARKb-mNG OX).

(F) Comparison of actin growth rates near the cell tip. F-actin was visualised using Lifeact-mNG. The mean rate was 1.79 ± 0.0790 µm/sec (ΔARKabc, ±SEM, n = 14), 2.07 ± 0.084 µm/sec (ΔARKabc/ARKb-mNG OX, ±SEM, n = 23). The P-value was calculated using Welch’s two-sample t-test: P = 0.02227 (ΔARKabc - ΔARKabc/ARKb-mNG OX).
Fig. 6 ARK and microtubules are necessary for the tip localisation of RopGEFs

(A) and (B) RopGEF3 and RopGEF6 levels at the cell tip were decreased in the ARK mutant. (A) Representative images of RopGEF3-mNG and RopGEF6-mNG at the cell tip. Scale bar, 5 µm. (B) Comparison of the intensity of RopGEF3-mNG and RopGEF6-mNG at the cell apex. The mean intensity of RopGEF3-mNG was 1.82 ± 0.202 (control, ±SEM, n = 6), 1.20 ± 0.0792 (ΔARKabc, ±SEM, n = 7). P-values were calculated using Welch’s two-sample t-test: P = 0.0119944 (control - ΔARKabc). The mean intensity of RopGEF6-mNG was 1.85 ± 0.0905 (control, ±SEM, n = 7), 1.23 ± 0.0825 (ΔARKabc, ±SEM, n = 10). P-values were calculated using Welch’s two-sample t-test: P = 0.000395 (control - ΔARKabc), P = 0.0000050 (ΔARKabc - ΔARKabc/RopGEF3-mNG OX).

(C) and (D) Representative images and comparison of For2A-mNG intensity at the cell tip. The mean intensity was 1.03 ± 0.0988 (control, ±SEM, n = 10) and 0.802 ± 0.0640 (ΔARKabc, ±SEM, n = 11). The P-value was calculated using Welch’s two-sample t-test: P = 0.06873 (control - ΔARKabc).

(E) Motor-RopGEF-fusion constructs used in this study. Tailless ARKb and mNeonGreen-FLAG were fused to the N-terminal and C-terminal sides of RopGEF3, respectively. Scale bar: 100 amino acids.

(F) Representative images of ΔARKabc moss expressing native or motor-fused RopGEF3 (ARKb(ΔTail)-RopGEF3-mNG). Top row: 5-week-old moss colonies grown from a single protoplast; second row: 6-day-old protonemata; third row: tip localisation of the fusion protein. Scale bars: 2 mm (top row), 200 µm (second row), and 5 µm (third row).

(G) Relative intensity of chloroplasts along the apical cell at 150 min after anaphase onset. Chloroplast accumulation near the basal cell wall observed in ΔARKabc was not rescued by the fusion RopGEF3 construct.

(H) Area comparison of 5-week-old moss colonies. Colonies were cultured on BCDAT medium from a single protoplast for five weeks. The mean area (mm²) was 28.2 ± 2.42 (ΔARKabc, ±SEM, n = 18), 50.5 ± 3.08 (ΔARKabc/RopGEF3-mNG OX, ±SEM, n = 18), 91.5 ± 4.41 (ΔARKabc/ARKb(Δtail)-RopGEF3-mNG, ±SEM, n = 18). P-values were calculated using Tukey’s multiple comparison test: P = 0.1878188 (ΔARKabc - ΔARKabc/RopGEF3-mNG OX), P < 0.0000001 (ΔARKabc - ΔARKabc/ARKb-RopGEF3-mNG).
**Fig. 7** AtARK2 and AtARK3 expression partially rescues moss ΔARKabc

(A) Representative images of 5-week-old moss colonies regenerated from a single protoplast (top) and 6-day-old protonemata (bottom). Scale bars: 2 mm (top) and 200 µm (bottom).

(B) Representative images of protonemal apical cells. Arrowheads and white lines indicate the positions of the nucleus and the cell plate, respectively. Scale bar, 100 µm.

(C) Relative intensity of chloroplasts along the apical cell at 150 min after anaphase onset (±SEM). The basal accumulation of chloroplasts in ΔARKabc was abolished after the ectopic expression of AtARK2 but not of AtARK3.

(D) Model for microtubule-dependent transport in *Physcomitrium patens* protonemal cells.

Giant organelles, such as nuclei and chloroplasts, are transported to the plus and minus ends of the microtubules by ARK and kinesin-14s (KCH and KCBP), respectively (i.e. tug-of-war). ARK also transports secretory vesicles, which are presumably passed to myosin XI at the cell tip for exocytosis. The proteins and cell wall materials required for tip growth are also candidate cargos for ARK, as ARK depletion severely suppresses polarised cell growth.
Supplemental Fig. 1 Establishment of *Physcomitrium patens* ARK mutants

(A) Representative images of 3-week-old moss colonies of ARK mutants. mCherry-α-tubulin was used as a control. Scale bar, 5 mm.

(B) Sequencing revealed frameshift mutations in the ΔARKabc and ΔARKabcd sequences used in this study (displayed as SnapGene sequence files).
Supplemental Fig. 2 ARK deletion does not affect microtubule dynamics

(A) Comparison of microtubule dynamics. Upper row, control (mCherry-α-tubulin) and ΔARKabc; lower row, control (GFP-α-tubulin/Histone-mCherry), and ΔARKabcd. The mean ± SEM, number of samples, and P-values are shown in Table S1.

(B) Comparison of microtubule orientation in apical cells based on EB1-Citrine tracking. The quantification method is described in the Methods section. The frequency of tip-directed movement (%) was 97.0 ± 1.16 (mCherry-α-Tubulin, ±SEM, n = 18) and 88.5 ± 2.61 (ΔARKabc, ±SEM, n = 8). The P-value was calculated using Welch’s two-sample t-test; P = 0.007365.
Supplemental Fig. 3 The truncation-rescue assay reveals the essentiality of ARM repeats

(A) Truncated ARKb-mNG constructs used in this experiment. The protein domains were predicted using NCBI’s conserved domain database (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and SMART (http://smart.embl.de).

(B) Area comparison of 3-week-old moss colonies. Colonies were cultured from a single protoplast for three weeks on BCDAT medium. The datasets of ΔARKabc/FL (full-length) are identical to those shown in Fig. 1C. The mean area (mm²) was 4.88 ± 0.651 (ΔARKabc/ΔARM, ±SEM, n = 18), 7.04 ± 1.07 (ΔARKabc/ΔTail, ±SEM, n = 18).

(C) Relative intensity of chloroplasts along the apical cell at 150 min after anaphase onset. The chloroplasts remained accumulated near the basal cell wall after the expression of the ΔARM or ΔTail construct.
Supplemental Fig. 4 ARK deletion affects cell length, but not the cell cycle duration or Rop4 localisation

(A) Comparison of cell cycle durations. The mean duration (h) was 7.43 ± 0.307 (control, ±SEM, n = 24), 7.58 ± 0.232 (ΔARKabc, ±SEM, n = 13), 6.93 ± 0.220 (ΔARKabc/ARKb-mNG OX, ±SEM, n = 36).

(B) Comparison of tip growth rates in the presence of 500 pM or 50 nM latrunculin A (LatA). The mCherry-α-tubulin line was used for drug treatment. The mean rate (µm/h) was 9.69 ± 1.20 (ΔARKabc, ±SEM, n = 5), 23.0 ± 1.00 (control), 16.4 ± 1.20 (500 pM LatA, ±SEM, n = 6), 9.64 ± 0.702 (50 nM LatA, ±SEM, n = 17).

(C) and (D) Representative images and comparison of intensities of functional Rop4-mNG at the cell tip. The mean intensity was 1.29 ± 0.024 (control, ±SEM, n = 6), 1.54 ± 0.140 (ΔARKabc, ±SEM, n = 11).

(E) The apical accumulation of RopGEF3 and RopGEF6 depends on the microtubules. Snapshots of the same cell before and 50-min after oryzalin addition are shown. DMSO was used as a control. mCherry-α-tubulin-expressing RopGEF3-mNG or RopGEF6-mNG was used in this experiment. Scale bar, 5 µm.

(F) Temporal changes in RopGEF3-mNG or RopGEF6-mNG intensity at the cell tip after drug addition. A decrease in localisation was observed after microtubule disruption with oryzalin. Intensities relative to those before drug treatment are plotted (±SEM, n = 6–13).
Supplemental Fig. 5 RNAi of ARKd in ΔARKabc causes abnormal outgrowth

(A) Representative image sequences of abnormal cell growth after ARKd RNAi induction by β-estradiol. Left, outgrowth in the apical cell; right, outgrowth in the subapical cell. Scale bars, 100 µm.

(B) Comparison of apical cell shape after 1 µM β-estradiol or control 0.1% DMSO treatment. The numbers shown in the bar graphs indicate the actual number of counted cells.