BREAKTHROUGH REPORT

A Fully Functional ROP Fluorescent Fusion Protein Reveals Roles for this GTPase in Subcellular and Tissue-level Patterning

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Short Title: Functional ROP patterns cells and tissues

One-sentence summary: Tagging ROP in the middle of the protein produces a functional protein that provides all ROP function in juvenile moss tissues and marks sites with weakened cell walls long before expansion occurs.

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ABSTRACT

ROPs are GTPases that regulate polarity and patterned wall deposition in plants. As these small, globular proteins have many interactors, it has been difficult to ensure that methods to visualize ROP in live cells do not affect ROP function. Here, motivated by work in fission yeast (Schizosaccharomyces pombe), we generated a fluorescent moss (Physcomitrium [Physcomitrella] patens) ROP4 fusion protein by inserting mNeonGreen after Glycine 134. Plants harboring tagged ROP4 and no other ROP genes were phenotypically normal. Plants lacking all four ROP genes comprised an unpatterned clump of spherical cells that were unable to form gametophores, demonstrating that ROP is essentially for spatial patterning at the cellular and tissue levels. The functional ROP fusion protein formed a steep gradient at the apical plasma membranes of growing tip cells. ROP also predicted the site of branch formation in the apical cell at the onset of mitosis, which occurs one to two cell cycles before a branch cell emerges. While FRAP studies demonstrated that ROP dynamics do not depend on the cytoskeleton, acute depolymerization of the cytoskeleton removed ROP from the membrane only in recently divided cells, pointing to a feedback mechanism between the cell cycle, cytoskeleton, and ROP.
INTRODUCTION

Cell polarity is an important process in eukaryotic development. In plants, development occurs in the absence of cell migration and thus requires exquisite control of cell polarity to properly pattern tissues throughout the organism. For example, the establishment of polarized membrane domains with the appropriate auxin efflux carriers sets up the organization of the root (Blilou et al., 2005; Kania et al., 2014; van Dop et al., 2020), while the proper positioning of membrane markers in developing leaves leads to normal stomatal patterning (Mansfield et al., 2018; Zhang et al., 2016; Houbaert et al., 2018). In addition to the complex polarity establishment found in tissues (Zhang and Dong, 2018), seed plants have several cell types, such as root hairs and pollen tubes that undergo highly polarized cell expansion, and this expansion underlies their function. Root hairs are important for nutrient uptake (Gilroy and Jones, 2000), while pollen tubes are critical for sexual reproduction (Chen et al., 2018). In non-flowering plants, polarized cell expansion, also known as tip growth, generates protonemata and rhizoids. Protonemata in mosses establish the plant, as this is the tissue that emerges from the spore, while rhizoids help to anchor the tissue to the soil in both mosses and liverworts (Rounds and Bezanilla, 2013; Shimamura, 2016).

In plants, ROP (Rho-of-plants) proteins are small GTPases that share sequence similarity with the Rho/RAC/CDC42 family of G-proteins present in all other eukaryotes (Hall, 2012). Rho family proteins have been extensively studied in mammalian and yeast systems and are known to be critical regulators of cell polarity (Etienne-Manneville and Hall, 2002). For at least the past 20 years, numerous studies have established that ROPs are master regulators of cell polarity in plants (Bloch and Yalovsky, 2013). In flowering plants, ROP is important for patterned cell wall deposition, including the development of tip-growing pollen tubes and root hairs (Craddock et al., 2012; Hwang et al., 2005; Lin and Yang, 1997; Lin et al., 1996; Gu et al., 2006). Besides tip growing cells, ROP is also important for plant development at the tissue level (Fu et al., 2002; Zhang et al., 2019; Lin et al., 2012; Foucart et al., 2009). The appropriate transcriptional regulation of ROP is essential for patterning the puzzle-shaped pavement cells in Arabidopsis thaliana (Lin et al., 2015; Fu et al., 2005; Xu et al., 2010). During secondary cell wall formation, Arabidopsis ROP11 induces the formation
of xylem pits via the local disassembly of microtubules (Sugiyama et al., 2019; Oda and Fukuda, 2013, 2012; Nagashima et al., 2018). Various studies have also discovered that ROP genes influence signaling processes involved in pathogen defense, stress responses, and nodule symbiosis in various species (Ke et al., 2012; Lei et al., 2015; Huang et al., 2014; Poraty-Gavra et al., 2013; Wang et al., 2018; Miao et al., 2018; Venus and Oelmüller, 2013). In contrast to flowering plants, which generally have multiple ROP genes grouped into three subfamilies (Bloch and Yalovsky, 2013), the model moss Physcomitrium (Physcomitrella) patens has four highly similar ROP genes within a single subfamily (Eklund et al., 2010; Ito et al., 2014). In fact, the translation of the four P. patens ROP genes results in the production of three distinct proteins that differ by at most two amino acids (ROP1 and 4 are identical; ROP1/4 differ from ROP2/3 by one amino acid; ROP2 differs from ROP3 by 2 amino acids). Loss of function studies demonstrated that these ROP genes are functionally redundant in controlling polarized growth (Burkart et al., 2015).

Like the majority of small G-proteins, many ROPs (including all four in P. patens) possess a C-terminal CAAX motif that is important for membrane targeting (Yang, 2002; Yalovsky, 2015; Lavy et al., 2002). The cysteine in the CAAX motif serves as a site for prenylation, creating a lipid anchor that can insert into the plasma membrane (Geyer and Wittinghofer, 1997). ROPs also bind to numerous effectors and are regulated by many interacting proteins. For example, guanine exchange factors (GEFs) trigger ROP to release GDP and bind to GTP. GTPase-activating proteins (GAPs) bind to GTP-ROP and activate its intrinsic GTPase activity. Guanine dissociation inhibitors (GDI) bind to GDP-ROP, interacting with the covalent lipid modification, thereby removing GDP-ROP from the plasma membrane. These proteins interact with ROP directly to precisely regulate its activation both spatially and temporally (Feiguelman et al., 2018; Bascom et al., 2019; Dovas and Couchman, 2005). As a result, the binding sites for these interacting proteins on ROP need to remain accessible for normal ROP function. ROP is a small protein of only 196 amino acids, which is smaller than commonly used fluorescent proteins. Thus, it is challenging to create a fluorescent ROP fusion protein in which all aspects of ROP function remain intact.
Because the covalent lipid modification occurs on the C-terminus of ROP, the vast majority of studies in plants have utilized N-terminally tagged ROP proteins. Inspection of ROP’s sequence and comparison to related G-proteins whose structures have been determined revealed that the ROP N-terminus is near the nucleotide binding pocket, the switch regions, and the interface known to interact with regulators such as GDI (Hoffman et al., 2000). The switch regions comprise the structural features that define the distinct conformations when ROP is bound to either GTP or GDP (Vetter, 2001). Given that several critical ROP features are near the N-terminus, tagging at the N terminus could possibly influence normal ROP function. N-terminally tagged Rho family GTPases have been commonly used for fluorescent live cell imaging (Wedlich-Soldner, 2003). However, in both budding and fission yeast, gene replacement studies demonstrated that the function of N-terminally tagged CDC42 was affected, introducing temperature sensitive growth defects (Howell et al., 2012; Bendezú et al., 2015) and negatively influencing CDC42’s association with exocytic vesicles (Watson et al., 2014). As an alternative to N-terminal tagging, Burkart et al. investigated the possibility of tagging ROP before the CAAX motif in *P. patens*. The authors introduced sequences encoding GFP immediately upstream of the CAAX motif in the endogenous *ROP4* genomic locus. However, tagging ROP in this manner was unable to rescue ROP function and could not drive polarized growth (Burkart, 2015).

Besides direct N-terminal tagging, reporter systems for labeling active ROP domains have utilized ROP binding proteins or their specific binding domain (CRIB motif) fused to a fluorescent protein in both animal and plant systems (Nalbant, 2004; Zhu and Fu, 2012; Li et al., 2018). Similarly, Förster resonance energy transfer (FRET) sensors that visualize ROP activation dynamically have also been developed (Wong et al., 2018; Liu et al., 2011; Itoh et al., 2002). These reporters are nonetheless based on the direct binding of the conserved CRIB motif (also GBD for GTPase binding domain or PBD for p21 binding domain) to the Rho family GTPase of interest. The CRIB motif is present in RhoGAPs (Schaefer et al., 2011; Wu et al., 2000) and GTPase effectors. The most well-studied effectors include PAK (p21-activated kinase), ACK (activated Cdc42-associated kinase), and WASP (Wiscott-Aldrich Syndrome protein) for CDC42 in animal and yeast systems (Hoffman and Cerione, 2000) and RIC for ROP in plant systems (Wu et al., 2000).
While these approaches are powerful because they focus on identifying subcellular domains containing the active GTPase, it can be difficult to ensure that all aspects of GTPase function remain unimpaired, since the unregulated expression of reporters could compete with regulators and effectors for the CRIB domain binding sites on the GTPases.

Expressing N-terminally tagged ROP has been the most common approach to study ROP localization in plants (Li et al., 2018, 1999; Yalovsky et al., 2008; Sun et al., 2015; Yi and Goshima, 2020a). Even with efforts to tune down the expression level with weaker or inducible promoters (Le Bail et al., 2019), the expression levels need to be carefully monitored to avoid introducing new phenotypes. Notably, N-terminally tagged ROP fusion proteins exhibit activity when expressed. For example, ROP with an N-terminal GFP tag induces the depolarization of pollen tube growth (Kost et al., 1999), which also occurs when untagged ROP is overexpressed (Fu et al., 2001). Overexpressing constitutively active ROP causes root hair swelling (Molendijk, 2001) and narrower leaf shape (Bloch et al., 2005). However, while these activities implicate function, they are not equivalent to demonstrating the replacement of the normal function of untagged ROP. Complementation studies similar to what has been carried out in budding and fission yeasts (Watson et al., 2014; Bendezú et al., 2015; Howell et al., 2012) have been challenging in plants. Arabidopsis has 11 ROP genes, many with overlapping functions (Craddock et al., 2012), making it difficult to perform clean genetic complementation in a flowering plant model system.

The moss _P. patens_ can be easily propagated vegetatively, and mutants can be recovered after targeted genome editing. Recent advances in CRISPR technology have provided excellent tools to simultaneously edit several genomic loci (Lopez-Obando et al., 2016; Collonnier et al., 2017; Mallett et al., 2019; Yi and Goshima, 2020b), making testing fluorescently fused ROP in a complete knockout background of the whole gene family feasible. In the current study, we inserted the coding sequence of mNeonGreen fluorescent protein into the native genomic locus of _P. patens ROP4_ without introducing any other alterations to the genome. We tested fusion proteins with the commonly used N-terminal tag and with an internal "sandwich" tag inspired by previous work in fission yeast with CDC42 (Bendezú et al., 2015). We determined that only the sandwich tagged
ROP4 fusion protein maintained full functionality and was sufficient to drive polarized
growth in the absence of all other ROP proteins. Using this functionally tagged ROP
fluorescent marker, we investigated ROP localization during growth and its dependence
on the cytoskeleton. We discovered that ROP polarization in growing cells is affected by
changes in the cytoskeleton in a cell cycle-dependent manner, revealing a potential
feedback mechanism between growth, polarity, and the cell cycle.

RESULTS

N-terminally tagged ROP4 is not functional

Previously, homologous recombination was used to replace the genomic region
containing the ROP4 open reading frame with either a 5' GFP-tagged cDNA or an
untagged cDNA (Burkart et al., 2015). ROP4 was chosen because it encodes the same
protein as ROP1. Furthermore, ROP4 and ROP3 exhibit similar expression levels and
are the two most highly expressed ROP genes in protonemata. Even though the GFP-
tagged protein was expressed, neither the GFP-tagged nor the untagged cDNA was
able to replace the function of the wild-type gene, suggesting that the genomic context
of the coding sequence is critical for ROP4 function. To minimally alter gene structure,
we used CRISPR-Cas9 mediated homology directed repair (HDR) (Mallett et al., 2019)
to insert sequences encoding mEGFP (Vidali et al., 2009) or three tandem mEGFPs
(3XmEGFP) immediately downstream of the ROP4 start codon (Supplemental Figure
1A). To determine if the ROP4 fusion protein was functional, we introduced the mEGFP
sequences into a line where the ROP4 3'UTR was deleted, rendering the ROP4 locus
insensitive to an RNAi construct targeting the 3'UTR (Burkart et al., 2015). Thus, using
RNAi, we could specifically silence the other three ROP genes that are functionally
redundant with ROP4 in the tagged line (Burkart et al., 2015).

Transforming the stably tagged lines with an RNAi construct targeting the coding
sequence regions of all ROP genes (CDS RNAi) and the nuclear GFP:GUS reporter
resulted in plants lacking nuclear GFP that were composed of small spherical cells (Fig.
1A). Transformation with a construct that targets the 3' untranslated regions of all ROP
genes (UTR RNAi) and the nuclear GFP:GUS reporter should not affect ROP4
expression, since the targeting sequence has been deleted from the ROP4 gene. Thus,
we would predict that if the tagged ROP4 is functional, plants transformed with the UTR-RNAi construct should exhibit polarized growth. In contrast to this prediction, plants transformed with the UTR RNAi construct were unpolarized and indistinguishable from the CDS RNAi transformed plants (Fig. 1A). These data demonstrate that the N-terminally tagged ROP proteins are not functional, as neither can maintain polarized growth in moss protonemata in the absence of all other ROP proteins.

**Internally tagged ROP4 fully rescues polarized growth**

Inspired by previous work in fission yeast (Bendezú et al., 2015) where the ROP homolog CDC42 was functionally tagged in the middle of the protein (sandwich tag), we set out to test whether a similar approach might work for ROP. The fission yeast CDC42 sandwich tag was inserted in a small stretch of amino acids after the α3’ helix. As a result, the fluorescent protein looped out of the protein away from interfaces known to mediate interactions with CDC42. We identified a small stretch of non-conserved amino acids in ROP4 at the analogous CDC42 insertion site and speculated that this region may not be essential for ROP protein function. To test this notion, we generated a sandwich tag for *P. patens* ROP4 by inserting the coding sequence of mNeonGreen (mNG) after Glycine 134 (Supplemental Figure 1B).

Using CRISPR-mediated HDR, we introduced sequences encoding mNG into the ROP4-Δ3’UTR reporter line, rendering the sandwich-tagged ROP4 locus insensitive to the UTR RNAi construct. Remarkably, in two independently generated sandwich-tagged lines, silencing with the UTR RNAi construct resulted in polarized plants (Fig. 1B) indistinguishable from the parental untagged line. Notably, plant size was reduced to the same extent in sandwich-tagged and untagged control lines (Fig. 1C), and solidity, a measure of plant polarity, was similar in all lines (Fig. 1D). These data demonstrate that the untagged and sandwich-tagged ROP4 (hereafter referred to as ROP4-swmNG) contribute equally to ROP function. As expected, silencing with the CDS RNAi construct in all lines resulted in unpolarized plants composed of circular cells with no developmental organization (Fig. 1B). The finding that the sandwich-tagged lines transformed with the control RNAi constructs were smaller than the untagged control
parental line might reflect differences in expression and/or stability of the sandwich-tagged fusion protein due to the deletion of the 3' UTR.

To control for possible expression effects resulting from deletion of the 3' UTR and to ensure that incomplete silencing of ROP did not account for the observed rescue of polarized growth, we isolated several lines where the ROP4 locus with an intact 3' UTR was tagged appropriately with swmNG and carried null mutations in ROP1, 2 and 3, indicating that ROP4-swmNG does not affect plant viability. While these lines carried different mutations (Supplemental Figure 2), they all grew normally. Some of the mutants were isolated after co-transforming moss protoplasts with the CRISPR-Cas9 construct and double-stranded DNA oligos containing an in-frame stop codon. These oligos were designed to integrate at the Cas9 cut site via HDR, ensuring that a stop codon would be present soon after the double-strand break (Yi and Goshima, 2020b).

One of the lines that incorporated the designed pre-mature stop codons at all three targeted ROP loci was chosen for subsequent experiments (ROP4-swmNG/Δrop1/2/3-196 [Supplemental Figure 2], hereafter referred to as ROP4-swmNG/Δrop1/2/3). As a control, we also generated the same null mutations in the wild-type background (Δrop1/2/3-198 [Supplemental Figure 2], hereafter referred to as Δrop1/2/3). As expected based on previous RNAi data ((Burkart et al., 2015), Fig 1B-D), Δrop1/2/3 plants were smaller than the wild type (Fig. 1E, F) but exhibited no defects in plant morphology, as measured by solidity (Fig. 1E, G). Remarkably, the sandwich-tagged ROP4 line grew similarly in the presence or absence of the remaining ROP genes (Fig. 1E, G), demonstrating that ROP4-swmNG is sufficient to drive polarized growth in P. patens protonemata.

Compared to the N-terminal fusion protein, ROP4-swmNG localizes to a smaller region of the growing tip

N-terminal ROP fusion proteins localize to the apical plasma membranes of pollen tubes (Li et al., 1999, 2018; Sun et al., 2015), root hairs (Yalovsky et al., 2008), and moss protonemata (Burkart et al., 2015; Yi and Goshima, 2020a). Since the N-terminal fusion protein is not functional, we wondered whether its localization might be affected. Thus, we used laser-scanning confocal microscopy to compare the localization
of the N-terminal or sandwich tagged ROP4 in growing moss protonemata. Similar to previous studies (Burkart et al., 2015; Yi and Goshima, 2020a), regardless of whether ROP4 was tagged with mEGFP or 3XmEGFP, the N-terminal fusion protein localized to the apical plasma membrane (Fig. 2A). Interestingly, the 3XmEGFP tag was not necessarily brighter on the plasma membrane. Instead, cells with this tag had a higher cytosolic background, which might result from cleavage of the N-terminal GFP tag, as mentioned in (Yi and Goshima, 2020a). Medial plane images of mEGFP-ROP4 revealed a strong plasma membrane signal that covered the entire apical dome of the cell. Maximum projections of confocal Z-stacks demonstrated that this signal often extended 15-20 µm back from the cell tip (Fig. 2A). While ROP4-swmNG was also enriched at the apical plasma membrane, regardless of the presence or absence of the other three ROP genes, ROP4-swmNG covered a much more tightly focused area of the cell tip. Maximum projections of Z-stacks demonstrated that the ROP4-swmNG signal only reached 5-10 µm back from the tip (Fig. 2A).

To quantify the differences between the plasma membrane localizations of the N-terminal and sandwich fusion proteins, we drew a line on the plasma membrane of medial sections of apical cells with normalized fluorescence. We obtained fluorescence intensity profiles of the plasma membrane and measured the width of the peak at the same intensity value for all cells (Fig. 2B). We discovered that GFP-ROP was spread out over a larger area of the cell apex compared to ROP4-swmNG (Fig. 2C), which is consistent with the maximum projections of confocal Z-stacks (Fig. 1A). We also fit a line to both the increase and decrease in fluorescence intensity and calculated the absolute value of the slope (Fig. 2B). ROP4-swmNG had significantly larger slopes than GFP-ROP (Fig. 2D), demonstrating that the sandwich tagged ROP also formed a steeper gradient on the plasma membrane. Together, these data demonstrate that the functional ROP4-swmNG localizes to a more restricted region of the cell apex than the N-terminal fusion protein.

During changes in growth direction ROP4-swmNG accumulates at sites of cell expansion
With a functional fluorescent fusion protein in hand, we investigated ROP dynamics during polarized growth. At a growing tip, the apical ROP4-swmNG gradient tracked with the growing tip as it elongated, and no obvious periodic fluctuations were observed in growing cells (Fig. 3A, Supplemental Movie 1). In a growing moss plant, not all protonemal filaments are actively growing. During long-term imaging, it is possible to occasionally observe cells that cease to grow and after some time resume growing. In one of these cells, ROP disappeared from the apical plasma membrane when the cell stopped growing (Fig. 3A, Supplemental Movie 1). After 100 minutes, ROP repopulated the left side of the membrane at the cell tip, and when the cell resumed growing, it turned towards the left, indicating that areas of ROP accumulation predict the site of cell expansion (Fig. 3A, Supplemental Movie 1). Unfortunately, predicting when a growth site will cease is challenging. Therefore, to examine the behavior of ROP during changes in the direction of growth and the establishment of new growth sites, we used an assay that produces directional changes as well as frequent pauses. When microtubules are disrupted with oryzalin, the growing tip randomly changes direction, and new tips occasionally emerge from the apical cell (Wu and Bezanilla, 2018). Using long-term oryzalin treatment as a tool, we were able to image moss protonemal filaments growing in microfluidic devices for up to 5 days with 12.5 µM oryzalin present in the growth medium. Even with multiple changes in growth direction, ROP consistently associated with the growing tip (Fig. 3B, C, Supplemental Movies 2 and 3).

We tracked the focused ROP spot with the TrackMate plugin in Fiji (Tinevez et al., 2017) to generate a trace for ROP movement, which aligned closely with the path of cell growth (Fig. 3B, C, Supplemental Movie 2). Interestingly, when the cell experienced a period of unfocused growth, we also observed diffuse ROP signal overlapping with the site of cell swelling (Fig. 3B, 96min, 771min, Supplemental Movie 2). Occasionally, we observed ROP accumulation in an area of the cell that was not expanding (Fig. 3C blue arrowhead). However, in this example (Fig. 3C, 375min), the signal soon decreased, and no growth occurred at that site (Supplemental Movie 3). New polarized growth sites also often formed independently from the original growth site. In this case, as the accumulation of ROP at the old growth site diminished, the cell stopped growing at that site. Concurrently, ROP accumulated near the new growth site where cell expansion
took off and a new tip emerged (Fig. 3C yellow arrowheads, Supplemental Movie 3).

These data demonstrate that ROP accumulates at actively growing sites. ROP accumulated most strongly during focused tip growth, while unfocused expansion was characterized by diffuse ROP signal along the membrane. Importantly, new sites of polarized expansion always exhibited a strong ROP signal, suggesting that ROP marks the site of maximal expansion.

**The cytoskeleton affects ROP polarity in a cell cycle-dependent manner**

As master regulators of polarity, ROP and related GTPases affect both actin and microtubule behavior in plants and other eukaryotes (Burkart et al., 2015; Gu et al., 2003; Sugiyama et al., 2019; Iden and Collard, 2008). To investigate whether ROP localization depends on actin, which is essential for polarized growth (Vidali et al., 2009), we tested whether acute treatment with latrunculin B, which disrupts the actin cytoskeleton, would affect ROP localization. First, we identified actively growing apical cells and infused them with 0.5% DMSO. All actively growing cells retained ROP localization at the cell tip (N=34 cells). By contrast, after infusion with latrunculin B, roughly 30-40% of the cells lost the apical ROP gradient (Fig. 3D). Infusion with oryzalin, which disrupts microtubules, also resulted in a similar fraction of cells that lost the apical gradient (Fig. 3D). To investigate why only a fraction of cells exhibited this behavior, we quantified the length of the apical cell and normalized it to the length of the subapical cell. Interestingly, short apical cells lost the apical ROP gradient in response to either latrunculin B or oryzalin, while long apical cells maintained this gradient (Fig. 3E). Since the apical cell is the dividing cell of the protonemal filament, the length of the cell is indicative of the stage of the cell cycle. The shortest cells are newly generated cells, whereas the longest cells are soon to enter cell division. Thus, these data suggest that actin and microtubules play a role in maintaining ROP localization early in the cell cycle.

To investigate if ROP dynamics at the cell cortex depend on the cytoskeleton, we used FRAP (fluorescence recovery after photobleaching) to examine ROP turnover in control cells and in cells that maintained the apical ROP gradient in the absence of the cytoskeleton. In the same 5-60 min time window after drug addition, cells that maintained normal ROP localization were photobleached at the cell tip (Supplemental
Movie 4). In both the \( \Delta rop1/2/3 \) (Supplemental Figure 3A) and wild type (Supplemental Figure 3B) backgrounds, the ROP signal readily recovered within 40 seconds (Supplemental Figure 3A, B black lines). Treatment with oryzalin (green lines) or latrunculin B (magenta lines) did not cause substantial changes in the rate or level of fluorescence recovery. Only latrunculin B treatment in \( \Delta rop1/2/3 \), but not in wild type, slightly decreased the rate of ROP recovery. As a whole, the FRAP data indicate that ROP4-swMNG mobility is not dependent on microtubules or actin in the wild type (Supplemental Figure 3, Supplemental Movie 4).

**Long-term growth inhibition mediated by actin depolymerization leads to changes in ROP4-swMNG localization**

For a population of cells, ROP accumulation at the tip was not influenced by the acute depolymerization of actin (Fig. 3D, E). To evaluate whether ROP remained associated with a former growth site in the absence of growth over long time periods, we treated protonemal tissue with 25 \( \mu M \) latrunculin B in a microfluidic imaging device. After identifying apical cells that retained tip-localized ROP accumulation within the first hour of treatment, we continued to image these cells for the next 16 h. Two hours after the addition of latrunculin B, most of the cells that had retained the ROP gradient within the first hour stopped growing completely. In addition, the apical ROP enrichment dissipated in many cells after two hours, eventually leading to a complete loss of the ROP signal. Interestingly, in a small population of cells, we observed intracellular membranous structures enriched with ROP (Supplemental Figure 4, yellow arrowheads). These membranous structures were morphologically diverse and dynamic, and their appearance seemed to correlate with the reduction of tip-localized ROP signal (Supplemental Figure 4, Supplemental Movie 5). Occasionally, we also observed smaller aggregates with ROP4-swMNG signal (Supplemental Figure 4, blue arrowheads). Similar to cells that naturally pause (Fig. 3A), these data demonstrate that in cells that stop growing due to the disruption of actin, the ROP signal disappears from the tip, suggesting that there is a feedback mechanism between growth and ROP localization to help maintain ROP at the site of polarized growth.
ROP is essential for developmental patterning, predicting the future branching site during the apical cell division

While generating ROP4-swmNG lines in the Δrop1/2/3 background, we isolated a number of transformants containing lesions in all four ROP genes (Supplemental Figure 5). Despite their extremely slow growth rates, all Δrop1/2/3/4 mutants could be maintained vegetatively indefinitely. Imaging growing Δrop1/2/3/4 plants revealed that their cells grew isotropically with no obvious branching pattern (Fig. 4, Supplemental Movies 6, 7). While cell division still generated a flat cell plate between daughter cells, the combination of subsequent isotropic expansion coupled with weak cell adhesion resulted in daughter cells that eventually became loosely associated spheres (Fig. 4, orange arrowheads) Furthermore, the Δrop1/2/3/4 mutants did not form gametophores. We also isolated a triple mutant, Δrop1/3/4 (Supplemental Figure 2), with an intact ROP2 gene that exhibited an intriguing intermediate phenotype. Apical cells grew slowly but were polarized. However, after cell division, subapical cells swelled and often divided aberrantly, resulting in protonemal filaments composed of rounded cells with few branches and elongated apical cells (Fig. 4, blue arrowheads, Supplemental Movie 8), which differed substantially from the other triple mutants (Fig. 1E, Yi and Goshima, 2020a) and wild-type protonemata (Fig. 4, red arrowheads, Supplemental Movie 6). In contrast to the complete null mutant, Δrop1/3/4 formed normal gametophores (Supplemental Figure 5C, Supplemental Movie 9). Taken together, these data suggest that proper ROP levels are essential for ensuring patterning at the subcellular level and at the tissue level.

During branch formation, protonemal filaments initiate a new site of polarized cell expansion. Given that Δrop1/2/3/4 plants did not appear to have clear branching patterns (Fig. 4, Supplemental Movie 7) and that Δrop1/3/4 plants exhibited improperly positioned cell plates in swollen subapical cells (Fig. 4, Supplemental Movie 8), resulting in aberrant branch patterning, we investigated the timing of ROP localization during branch formation. ROP4-swmNG localized to the newly formed cell plate in apical cells (Fig. 5A), similar to observations made with the non-functional N-terminal ROP4 fusion protein (Burkart et al., 2015; Yi and Goshima, 2020a). However, in addition, we discovered that ROP actually accumulates at the cell cortex in the middle of the apical
cell 34 ± 10 minutes (mean ± standard deviation, N=18 cells) before it is observed in the developing cell plate (Fig. 5A white arrows, Supplemental Movie 10). Interestingly, this timing is consistent with the onset of mitosis. ROP remained at the cell cortex for 307 ± 77 minutes (mean ± standard deviation, N=9 cells), with one side accumulating more ROP signal than the other. The cortex with the strongest ROP signal initiated tip growth and a new branch emerged (Fig. 5A cyan arrows, cyan lines in kymograph, Supplemental Movie 10). ROP was also found at the cell cortex across from the branch site marking the site of emergence of a future second branch (Fig. 5A cyan arrows).

These data demonstrate that ROP predicts the site of branch formation long before cell expansion occurs at the site.

Finally, we wondered whether ROP localization is associated with cell wall patterning prior to expansion. We reasoned that areas with the weakest cell wall would be the most sensitive to enzymatic digestion. Thus, we treated growing protonemal filaments in a microfluidic imaging device with Driselase, a cell wall digesting enzyme mix used to generate moss protoplasts, allowing us to image where protoplasts emerge from the protonemal filament. As expected, protoplasts extruded from the tip of the apical cell where the cell wall is the weakest (Fig. 5B white arrows, Supplemental Movie 11). Surprisingly, 47% of the time (N=15 cells), protoplasts were expelled from subapical cells with no obvious branch protrusions (Fig. 5B black arrows, Supplemental Movie 11) at the site where we had observed ROP accumulation during branching. By contrast, 53% of the time, the protoplast emerged from the apical cell plate or a position on the side of the filament more basal to the expected future branching site or did not emerge at all. Various factors could contribute to the inability of every protoplast to emerge from a future branch site. The weak cell wall might be positioned facing the cover slip or the top of the imaging chamber, preventing the protoplast from escaping. Alternatively, during normal protonemal development, perhaps not all subapical cells are programmed to branch, and thus weakening of the wall may not have occurred. Nevertheless, the finding that the site of protoplast emergence occurred where cortical ROP accumulated during branching suggests that ROP activity in these cortical domains might modify the cell wall long before cell expansion initiates. These data, together with the observation of ROP at normal and ectopic sites of expansion (Fig. 3A-
C), demonstrate that ROP is recruited to growth sites early on and likely participates in activating cell wall remodeling, ultimately promoting polarized cell expansion.

**DISCUSSION**

GTPases from the Rho/RAC/CDC42 family are relatively small and have many effectors and regulators that bind to different surfaces of these protein (Hodge and Ridley, 2016; Berken and Wittinghofer, 2008; Bascom et al., 2019). As such, it has been challenging to develop live-cell imaging tools that do not interfere with GTPase function. In seed plants, functional studies have further been complicated due to the presence of multiple redundant small GTPase family members. In contrast to seed plants, moss only requires a single ROP protein to drive growth during the juvenile stages of development (Burkart et al., 2015), providing a system to test the functions of ROP fusion proteins. Here, we showed that the most commonly used fusion protein, an N-terminally tagged ROP, is not functional, as it does not support growth when it is the only ROP present. However, inserting mNG into a loop downstream of Glycine 134 in ROP4 resulted in a fully functional protein, as plants containing only the tagged ROP4 grew normally and developed juvenile tissues indistinguishable from those of the wild type.

With this powerful tool in hand, it was possible to probe functional ROP dynamics and localization during growth and development. Here, we found that ROP localizes to the apical plasma membrane of tip-growing protonemata. The highest signal was detected at the tip, and it sharply declined moving away from the tip. During growth, the ROP signal was persistent, with no obvious fluctuations at the cell tip. While the N-terminal ROP fusion protein exhibited a similar localization pattern (Fig. 2, (Burkart et al., 2015; Yi and Goshima, 2020a)), it was quantitatively different, with a shallower tip gradient and populating a larger area of the cell apex. These results suggest that the functional sandwich-tagged fusion protein preferentially associates in a more restricted manner with the plasma membrane of actively growing cells.

Here, we found that ROP localization to the cell apex depends on actin and microtubules in cells that have just completed cell division. However, in cells that are closer to cell division, the acute loss of either microtubules or actin (5-60 min) does not affect ROP localization at the plasma membrane (Fig. 3). Additionally, our FRAP studies
showed that, similar to findings in root hairs (Molendijk, 2001), which are fully
differentiated, the turnover of ROP at the plasma membrane is largely independent of
the cytoskeleton. These results support the finding that in yeast, rather than the
cytoskeleton, differences in the diffusion rates of the active and inactive forms of G-
protein are critical for concentrating active G-proteins (Bendezú et al., 2015; Woods et
al., 2016). If actin depolymerization induces a stress response in moss, similar to what
occurs in yeasts (Harrison et al., 2001), perhaps the stress response is cell cycle
dependent. Early on in the cell cycle, the stress response is robust, ensuring that ROP
is removed. However, in a cell that has passed a threshold size or a particular phase in
the cell cycle and is committed to divide, ROP polarity is maintained so that the cell
continues to grow until it divides. With sustained actin depolymerization, stress
responses may result in changes in gene expression that alter potential cell fates,
leading to the removal of ROP from the apical plasma membrane (Supplemental Figure
4).

In contrast to long-term actin depolymerization, long-term treatment with
microtubule-depolymerizing drugs does not completely inhibit growth. In cells with
reduced levels of cytoplasmic microtubules, the persistent apical actin focus that
predicts the site of cell expansion behaves erratically, disassembling and assembling in
random spots throughout the cell. Wherever this actin spot is, cell expansion occurs
(Wu and Bezanilla, 2018). This assay provides a mechanism to observe ectopic sites of
cell expansion. Similar to cells that naturally pause and resume growth, we found that
ROP localizes to actively expanding regions where maximal cell expansion occurs. This
behavior likely reflects a conserved function of ROP, since it occurs in diverse tip
growing cells, from the protonemal stem cell in mosses to fully differentiated pollen
tubes in angiosperms (Luo et al., 2017). When an ectopic site emerges, ROP is found at
that site. Often when ROP localization is diffuse, the area of expansion is larger, but as
the expansion focuses and forms a tip, ROP localization also focuses, demonstrating
that membrane domains containing ROP strongly correlate with active growth sites (Fig.
3). A reduction in cytoplasmic microtubule levels disrupts growth directionality, but the
actual growth machinery, which depends on actin, is not inhibited.
During branch formation, a physiologically relevant growth initiation process, we found that ROP marks the future expansion site several hours before tip growth occurs (Fig. 5A, Supplemental Movie 10). In fact, based on this timing, ROP is recruited to the cell cortex in the apical cell just prior to mitosis, implying that branch patterning is established one to two cell cycles before branch emergence. During division of the apical cell, ROP moves from the cell cortex to the phragmoplast. After division is complete, ROP again is enriched at the cortex adjacent to the new cell plate, but only on the subapical cell side. Cell wall remodeling likely occurs here, as we discovered that the cell wall is frequently weakest at these sites (Fig. 5B, Supplemental Movie 11).

These findings suggest that ROP is likely required to activate effectors that remodel the wall before tip growth can occur. This mechanism is similar to what occurs at the tips of growing pollen tubes (Luo et al., 2017). However, in contrast to pollen tubes, which are terminally differentiated cells that predominately respond to external signals from the ovule, the recruitment of ROP in protonemata during apical cell division provides a temporal and spatial mechanism linking branch patterning to the cell cycle and ensuring that branches emerge proximal to a cell plate. Correct branch patterning guarantees the appropriate developmental organization and is thus important for the spreading out of moss protonemata in the environment. Future work will focus on identifying how ROP is recruited before mitosis in the apical cell and what effectors ROP activates to remodel the cell wall.

The functional ROP fluorescent fusion protein developed in this study could be used to analyze ROP localization and dynamics throughout development in a variety of plant species. Unlike CRIB4-GFP (Li et al., 2018), this functional fluorescent fusion protein does not necessarily identify active ROP populations. However, by using the ROP fluorescent fusion protein coupled with functional fluorescent GEF fusion proteins, it may be possible to image ROP activation domains on the plasma membrane. By analyzing ROP dynamics with the functional sandwich fusion protein in a variety of plant systems and comparing this to studies in animals and yeast, it may be possible to identify evolutionarily conserved mechanisms that regulate small GTPase localization and function.
METHODS

Plasmid construction and genotyping

To insert sequences into the genome using CRISPR-Cas9-mediated HDR, two plasmids were generally used: one plasmid contained the Cas9 and the protospacer expression cassettes for introducing double-stranded breaks at the desired genomic locations, and the other plasmid contained the sequence to be inserted and homology regions on either side of that sequence. For N-terminal tagging and for sandwich tagging, protospacers targeting the \textit{ROP4} genomic region near the desired insertion site (ATG for the N-terminal tag, and the codon for Glycine 134 for the sandwich tag) were incorporated into pMH-ROP4-N and pMH-ROP4-sw as described in (Mallett et al., 2019). To generate the homology plasmid for the N-terminal tag of \textit{ROP4}, primers DC334-337 (Supplemental Table 1) were used to amplify 800-1000 bp of genomic sequence up- and downstream of the protospacer target site (5' and 3' homology arms). The 2 homology arms were cloned into pDONR vectors and recombined with mEGFP or 3XmEGFP (Vidali et al., 2009) into a plasmid backbone derived from pGEM-T Easy (Promega), using a 3-way recombination reaction (Invitrogen) as described in (Mallett et al., 2019). To generate the homology plasmid for the sandwich tag, primers DC773-778 were designed to amplify 5' and 3' homology arms. A fragment of the mNG coding sequence was amplified by DC763,764. For the sandwich tag, we incorporated a linker as described previously for CDC42 (Bendezú et al., 2015). The homology plasmid was assembled by fusing the 5' homology arm, mNG, and the 3' homology arm into a plasmid backbone derived from pGEM-T Easy (Promega), using NEB HiFi assembly (New England Biolabs). Because the protospacer-targeted sequence was present in the homology plasmid, we introduced a point mutation at the third nucleotide of the protospacer adjacent motif (PAM) sequence in the homology arm using primers DC771,772.

To generate null mutations in \textit{ROP1, 2, and 3}, we constructed a pMH-Cas9-gate plasmid with three protospacers targeting the three genes specifically, according to (Mallett et al., 2019). First, the three protospacers were synthesized as complimentary oligos and annealed to each other. They were ligated into three different entry clones and recombined into pMH-Cas9 using a 3-way recombination reaction (Invitrogen) to
create pMH-ΔROP123. A plasmid with two protospacers targeting a single ROP1 at two different positions (pMH-ROP1-ps23) was constructed in a similar manner. Primers DC421-486 were used to genotype N-terminal tagged ROP4. Genotyping of ROP4-swmNG was done using primers DC634,635,767,768. For ROP knockout plants, genotyping was done using competition PCR (Harayama and Riezman, 2017) with primers DC1195-1434 and DC1185,1186. We were able to isolate transformed plants in which all three loci had incorporated the oligo sequences by HDR at very low frequency. However, at high frequency, we isolated mutants with one or two loci edited. To generate more mutants, we either transformed pMH-ROP123 again into single or double mutants or pMH-ROP1-ps23 into lines with the ROP1 and 2 loci mutated and selected plants with a deletion between the two protospacers.

Moss culture, transformation, protoplast regeneration, and RNAi assays

Physcomitrium (Physcomitrella) patens lines were propagated by homogenization with a tissue homogenizer (Omni International) and cultured on PpNH₄ medium (1.03 mM MgSO₄, 1.86 mM KH₂PO₄, 3.3 mM Ca(NO₃)₂, 2.72 mM (NH₄)₂-tartrate, 45 µM FeSO₄, 9.93 µM H₃BO₃, 220 nM CuSO₄, 1.966 µM MnCl₂, 231 nM CoCl₂, 191 nM ZnSO₄, 169 nM KI, and 103 nM Na₂MoO₄) supplied with 0.7% agar. After homogenization, 5 to 7-day-old tissue was used for protoplast transformation. For CRISPR-Cas9 mediated HDR, 7.5µg of the homology plasmid and 7.5 µg of the Cas9/protospacer plasmid were co-transformed into moss protoplasts as previously described (Liu and Vidali, 2011). To generate mutations in the ROP genes, we transformed 10-15 µg of pMH-ΔROP123 plasmid and also incorporated 5 µL of each of the three 50-60 bp double-stranded homology oligos (Supplemental Table 1) at 50 mM concentration. These oligos were homologous to the region adjacent to the protospacer, with a few mismatches deleting the PAM sequence and inserting an in-frame stop codon, adapting the methods described in (Yi and Goshima, 2020b). For transformation with pMH-ROP1-ps23, 15 µg of plasmid was used. To generate plants expressing Ubi:NLS-GFP-GUS, 60 µg of linearized pTK-Ubi:NLS-GFP-GUS (generated according to (Wu and Bezanilla, 2014)) plasmid was transformed in wild type and selected with...
20 µg/mL G418. Plants with stable integration of the G418 antibiotic resistance and exhibiting clear nuclear-localized GFP were selected.

Transformed protoplasts were plated with liquid plating medium made of PpNH₄ supplemented with 8.5% mannitol and 10 mM CaCl₂ onto cellophane-covered PRM-B plates (PpNH₄ medium plus 6% mannitol and 10 mM CaCl₂). After a 4-day regeneration period, the plants were transferred to PpNH₄ plates by moving the entire piece of cellophane. For growth assays, normal PpNH₄ was used, and for transient RNAi silencing and stable genomic editing, PpNH₄ plates supplemented with with 15 µg/mL Hygromycin were used. For transient RNAi silencing transformations, plants were imaged 7-days after transformation and discarded. For genomic editing stable transformations, plants were kept on PpNH₄ with 15 µg/mL Hygromycin selection plates for a week and transferred to normal PpNH₄ plates to allow more tissue to be grown for genotyping.

RNA silencing experiments using the ROP4-∆3'UTR reporter line with a nuclear localized GFP:GUS (NLS-GFP:GUS) in its genome were performed according to (Bezanilla et al., 2003; Burkart et al., 2015). Silencing constructs were the same as described in Burkart et al. N-terminal and sandwich tagged ROP4 were stably transformed and incorporated at the ROP4 locus into the ROP4-Δ3'UTR/NLS-GFP reporter background to create the tagged plants. Together with the untagged line as a control, 5 to 7-day-old ground tissue was used to generate protoplasts, which were transformed with RNA silencing constructs. The control construct targets the NLS-GFP transcript, the CDS-RNAi construct targets all 4 ROP transcripts within their coding sequences, and the UTR-RNAi construct targets all 4 ROP transcripts at their 3'UTRs, but ROP4 is insensitive to this construct, as its 3'UTR sequences were removed using homologous recombination. Seven days after transformation, plants regenerated from single protoplasts were imaged. Plants lacking NLS-GFP:GUS signal were successfully silenced and were imaged by capturing the chloroplast autofluorescence. A Nikon SMZ25 stereomicroscope with a filter cube (excitation 480/40, dichroic 510, emission 510 long pass) and a color camera (Nikon digital sight DS-Fi2) was used.

To quantify the size and polarity of ROP null mutant plants, 5 to 7-day-old ground tissue was used to generated protoplasts. Plants were regenerated from protoplasts on
PRM-B plates for 4 days, followed by PPNH₄ plates for 3 days. Seven days after generating protoplasts, individual plants were stained with 0.1 mg/mL calcofluor staining solution on a microscope slide covered with a coverslip and imaged under a Nikon SMZ25 stereomicroscope with a violet filter cube (excitation 420/25, dichroic 455, emission 460 long pass). Images of either chloroplast autofluorescence (red channel) or calcofluor fluorescence (where the combined signal from the red/green/blue channels in the RGB image was converted to a single red channel with Fiji software) were used to analyze plant size and polarity according to (Vidali et al., 2007). In brief, images of single plants were manually cropped and highlighted by thresholding the fluorescent signal with Fiji software (Schindelin et al., 2012). Total plant area and circumference were determined from the thresholded images to calculate area and solidity.

**Bright-field microscopy**

To observe plant growth, we loaded plant tissue into a PDMS microfluidic imaging device attached to a glass bottom dish (Bascom et al., 2016) filled with Hoagland’s liquid medium. Wild type and Δrop1/3/4 plants were homogenized before loading as described in (Bascom et al., 2016). However, because Δrop1/2/3/4 cells were not tightly adhered to one another, no homogenization was required before loading. Time-lapse images were acquired every 10, 20, or 30 minutes. At each time point, multiple positions in the imaging field were imaged. For Δrop1/2/3/4, a 5-image Z-stack of slices 4 μm apart was captured at each position. For Δrop1/3/4 gametophore, a 3-image Z-stack of slices 2 μm apart was captured. A single image for each position was captured for all other tissue. Extended depth of focus (EDF) images were created for each Z-stack using Nikon NIS elements. Bright-field illumination light was kept on during the entire acquisition process to provide light for plant growth. Mono-color images were acquired with a Nikon DS-Qi2 camera. Colored images were acquired with a Nikon DS-Vi1 or Nikon digital sight DS-Fi2 cameras.

**Confocal microscopy**

For confocal imaging, moss tissue was homogenized and loaded into PDMS devices. After 3-5 days of recovery in constant light, actively growing tip cells on newly
formed filaments were identified for imaging. The PDMS devices were mounted on a Nikon A1R laser-scanning confocal microscope with a 1.3 NA 40× or 1.49 NA 60× oil immersion objective (Nikon). Laser illumination at 488 nm was used for mNG/GFP and chlorophyll autofluorescence excitation. Emission filters were 525/50 nm for mNG/GFP. Chlorophyll autofluorescence was collected at 680 nm after passing through a long-pass dichroic mirror, allowing wavelengths larger than 640 nm through. Time-lapse, z-stack, and multipoint image acquisition was controlled by NIS-Elements software (Nikon). For long-term imaging of branch initiation events, PDMS devices containing tissue with established actively growing apical cells were placed in far red light for 3-4 days prior to imaging. Far red light inhibits branching, but upon exposure to white light, newly growing apical cells branch very regularly, greatly increasing the success rate of capturing branch formation events. Image acquisition occurred at room temperature. White light from the LED brightfield light source was kept on for plant growth between image acquisitions.

Cell wall digestion and drug treatments

Tissue was cultured in PDMS imaging devices for at least 4 days to allow actively growing protonemal tissue to become established. For cell wall digestion, immediately before imaging on a laser-scanning confocal microscope, 0.5% Driselase (Sigma) in 8.5% mannitol solution was injected into the PDMS device. For pharmacological treatments, Hoagland’s medium was supplemented with 12.5 µM oryzalin or 25 µM latrunculin B and injected into the PDMS device prior to imaging. Within 5-10 min after drug injection, apical cells and their corresponding sub-apical cell lengths were measured using the segmented line measurement tool in NIS-Elements (Nikon). Meanwhile, z-stacks of each apical cell were acquired to determine changes in ROP localization.

To measure ROP signal intensity, the single slice image that best represented the medial focal plane was selected for each cell with Fiji software (Schindelin et al., 2012). A region of interest (ROI) was cropped for each image to isolate the same area at the tip of each cell. A segmented line was then manually drawn tracing the plasma membrane at the tip. Intensity values for each pixel along the segmented line were measured. To measure the peak width and slope in Figure 2, individual images were
normalized with the enhance contrast function in Fiji with 0.1% saturated pixels before measuring the value along the line. To determine whether the ROP signal was maintained after drug treatment in Figure 3, we measured the image intensity along the manually drawn trace (without normalization). All values along this line were normalized to the largest intensity value along the segmented line and plotted against segmented line length to generate the graph shown in Figures 2 and 3. “Loss” or “maintain” ROP tip gradient was determined manually based the shape of the curve in the graph of each cell. The long-term time-lapse videos were acquired beginning at 1 hour after drug injection. Tracking of ROP tip accumulation in the oryzalin-induced curvy cells was done using the TrackMate plugin in Fiji (Tinevez et al., 2017), with a spot diameter of 15 µm.

**FRAP assay**

Actively growing apical cells were identified in a PDMS device using a Nikon A1R laser-scanning confocal microscope with a 1.49 NA 60× oil immersion objective (Nikon). A circular stimulation ROI with a diameter of 2.4 µm was placed at the apical plasma membrane where the ROP signal was the strongest. The 408 nm laser at 50% power was used for stimulation. Before stimulation, images were taken every 1 s for 5 s, followed by stimulation for 1 s, and the imaging continued after stimulation every 1 s for a total of 1.5 min. The mean fluorescence intensity of the ROP-swmNG signal was measured within the stimulation ROI. For each cell, the mean intensity measurements for the first five time points before stimulation were averaged to generate the reference intensity, and the mean intensity value of every time point in the whole movie was divided by the reference intensity value to create a normalized mean intensity. Graphs were generated by averaging the normalized mean intensity of each cells and plotted versus time. For photobleaching during drug treatments, cells that maintained normal ROP tip localization within 1 hour of drug injection were identified and photobleached.

**Statistical analysis**

All statistical analyses were performed using KaleidaGraph software (Synergy Software). To compare plant sizes and solidity of 7-day-old plants regenerated from protoplasts (Figure 1), all analyses were done using One-way ANOVA with post-hoc
Tukey HSD test. P values smaller than 0.05 in the Tukey HSD test were reported as significantly different groups (Supplemental Tables 2-5). To compare the quantification of the tip gradient signal peak width and slope (Figure 2), Student t Test for unpaired data with equal variance was used, and P values smaller than 0.05 were determined as significant (Supplemental Tables 6-7).

Accession numbers
Sequence data from this article can be found in Phytozome under the following accession numbers: *ROP1*, Pp3c14_4310; *ROP2*, Pp3c2_20700; *ROP3*, Pp3c1_21550; *ROP4*, Pp3c10_4950.

Supplemental Data
Supplemental Figure 1. N-terminal and sandwich-tagging strategies.
Supplemental Figure 2. Genomic edits in *ROP* mutants.
Supplemental Figure 3. FRAP analysis of *ROP4*-swmNG.
Supplemental Figure 4. Long-term latrunculin treatment reveals aberrant *ROP* localization.
Supplemental Figure 5. Genomic edits in the *ROP4* locus in Δrop1/2/3/4 mutants.
Supplemental Table 1. Primers used in this study.
Supplemental Table 2. One-way ANOVA for Figure 1C.
Supplemental Table 3. One-way ANOVA for Figure 1D.
Supplemental Table 4. One-way ANOVA for Figure 1F.
Supplemental Table 5. One-way ANOVA for Figure 1G.
Supplemental Table 6. Student t Test for unpaired data with equal variance for Figure 2C.
Supplemental Table 7. Student t Test for unpaired data with equal variance for Figure 2D.
Supplemental Movie 1. ROP localization in a growing apical cell.
Supplemental Movie 2. ROP localization is focused during tip growth but diffuse during cell swelling in oryzalin-treated protonemata.
Supplemental Movie 3. ROP localization correlates with the direction of growth and newly formed growth sites in oryzalin-treated protonemata.

Supplemental Movie 4. Fluorescence recovery after photobleaching of ROP4-swmNG at the cell apex.

Supplemental Movie 5. Formation of ROP-enriched membrane structures in growth-inhibited cells exposed to long-term latrunculin B treatment.

Supplemental Movie 6. Tip growth and branching cell division in wild-type protonemata.

Supplemental Movie 7. Abnormal growth and development in \( \Delta rop1/2/3/4 \).

Supplemental Movie 8. Abnormal branching and cell division pattern in \( \Delta rop1/3/4 \).

Supplemental Movie 9. Normal gametophore development in wild type and \( \Delta rop1/3/4 \).

Supplemental Movie 10. ROP4-swmNG localizes to the future branch site hours before a branch emerges.

Supplemental Movie 11. ROP4-swmNG localization associates with areas of weakened cell walls.

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AUTHOR CONTRIBUTIONS

X.C. and M.B. designed the research. X.C., B.W.M., and S.R.C.S. performed the research. X.C. and M.B. wrote the article.

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Figure 1. ROP4 is functional when the fluorescent protein is inserted in a loop within the protein. (A-B) Transient RNAi assay performed in a P. patens line containing a nuclear GFP:GUS reporter and a deletion of the ROP4 3’UTR, which renders the ROP4 gene insensitive to the UTR RNAi construct as described in (Burkart et al., 2015). All RNAi constructs simultaneously silence the nuclear GFP:GUS reporter and the target genes, and the control construct only silences GFP:GUS. (A) Representative chlorophyll autofluorescence (red) images of 7-day-old plants regenerated from protoplasts. Control and N-terminally tagged ROP4 lines were transformed with the indicated RNAi constructs. Chlorophyll autofluorescence labels the plant, while loss of nuclear GFP signal indicates successfully silenced plants. Scale bar for all images, 100 µm. (B) Representative chlorophyll autofluorescence images of 7-day-old plants regenerated from protoplasts from control and sandwich tagged ROP4 (ROP4-swNG) transformed with the indicated constructs. Scale bar for all images, 100 µm. (C) For the RNAi experiments represented in B, the area of the chlorophyll autofluorescence was normalized to the chlorophyll autofluorescence area of plants transformed with the control plasmid. (D) Solidity (Convex hull area/area) for RNAi experiments represented in B. Numbers in parentheses indicate the number of plants for each group. Legend of the graph in (C) also applies to (D) and indicates that black, blue, and purple represent data from protoplasts transformed with the control, CDS, or UTR RNAi constructs, respectively. (E) Representative images of 7-day-old plants stained with calcofluor and regenerated from protoplasts from wild type, ∆rop1/2/3, and ROP4-swNG in wild type and ∆rop1/2/3 backgrounds. Scale bar, 200 µm. (F) Normalized area and (G) solidity for the sandwich tagged ROP4 in wild type (black) and ∆rop1/2/3 (magenta). Based on the Kolmogorov-Smirnov Test, the data in (C, D, F, G) are normally distributed. Different letters in (C, D, F) indicate groups with significantly different means, as determined by ANOVA with a Tukey’s HSD all pair comparison post test (α=0.05). Also see Supplemental Tables 2-5.
Figure 2. Comparison of the localization of the N-terminal and sandwich-tagged ROP4 proteins.
(A) Representative confocal images of growing moss protonemata of the indicated genotypes. Medial plane shows a single focal plane at the middle of the cell; MaxIP shows the maximum projection of a Z-stack taken through the entire cell volume. Scale bar for all images, 3 µm. (B) Example image and measurement of ROP4-swNG fluorescent signal along the plasma membrane. A 3 pixel-wide trace (magenta) of the plasma membrane was manually drawn and the normalized signal intensity along the line was plotted. Scale bar, 3 µm. (C) Measurement of the peak width (indicated by the horizontal gray line in B) normalized to the cell width measured 10 µm from the cell tip. N=7, GFP-ROP4; N=19, ROP4-swNG. (D) Average of the slopes (slope 1 and 2 in B, green dashed lines). Slopes were determined by fitting a line to the points in the increasing and decreasing segment of the curve. Statistical analyses were performed using Student t test for unpaired data with equal variance with t probability indicated in the graph. Also see Supplemental Tables 6-7.
Figure 3. ROP4-swmNG enrichment anticipates cell expansion, and depends on the cytoskeleton in short apical cells. (A) Time-lapse imaging of a ROP4-swmNG/WT apical protonemal cell growing and pausing. Yellow arrows indicate growth direction, yellow dashed line indicates pause in growth. Scale bar for all images, 5 µm. Also see Supplemental Movie 1. (B-C). Time-lapse acquisitions of ROP4-swmNG/WT protonemal cells treated with 12.5 µM oryzalin. Images were acquired in cells that grew from 1 to 120 hours after drug addition. To accurately track the ROP4-swmNG signal, chloroplast autofluorescence was removed by subtracting the value of a fraction of chlorophyll autofluorescence intensity (*0.4 for B and *0.2 for A and C) from the mNG intensity in the maximum projections of confocal z-stacks. Red traces were generated by TrackMate using the processed images to track the ROP4-swmNG signal at the growing tip, which mirrored the growth pattern of the cell. (B) Example of one cell switching between isotropic and polarized growth. The images at the top are the first and last time points, with the trace in red of the mNG signal for the entire time course. Four distinct events are depicted in this time course: isotropic swelling (96-333 min), polarized growth with small changes in direction (345-582 min), large change in direction (627-732 min), and pause in growth (771-912 min). The first time points for each of these events are shown on the left and are maximum projections of z-stacks. The right panel shows the overlay of the ROP4-swmNG enrichment in the first time point (gray), with a projection of the subsequent time points (every 3 min) for the subsequent period of time indicated in the lower right corner of each image (magenta). Scale bar for all images, 10 µm. Also see Supplemental Movie 2. (C) Example of one cell continuously turning (0-471 min) and later developing a new polarized growth site (yellow arrowheads). Blue arrowhead indicates ectopic accumulation of the ROP4-swmNG signal that later diminished and did not lead to subsequent growth. Scale bar for all images, 10 µm. Also see Supplemental Movie 3. (D) Example confocal images of ROP4-swmNG/WT, and signal intensity quantification of protonemal apical cell tips after drug treatment. Graphs were generated by measuring the signal intensity along a manually drawn line tracing the plasma membrane of the cell. Scale bar for all images, 3 µm. (E) Quantification of the ratio between apical to sub-apical cell length for cells that lost or maintained the apical ROP gradient after drug treatment. Protonemal cells from ROP4-swmNG/WT were exposed to 25 µM latrunculin B or 12.5 µM oryzalin for 5-60 minutes and were imaged using confocal microscopy. Cell lengths were measured during image acquisition. N=12, 6, 12, 8 for each category, respectively.
Figure 4. ROP levels influence polarized growth and developmental patterning. Bright field images of wild type, Δrop1/3/4 and Δrop1/2/3/4 from time-lapse acquisitions of growing protonemata. Red arrowheads in wild type indicate normal branch divisions, blue arrowheads in Δrop1/3/4 indicate abnormal cell divisions, and orange arrowheads in Δrop1/2/3/4 indicate events of diffuse growth forming spherical cells after previous cell division. Scale bars for all images, 20 µm. Also see Supplemental Movie 6, 7 and 8.
Figure 5. ROP4-swNG predicts the site of branch formation five hours before branches emerge. (A) Maximum intensity projections of confocal Z-stacks of a growing protonemal filament in ROP4-swNG. Scale bar for all images, 10 µm. Also see Supplemental Movie 10. Green, ROP4-swNG signal; Gray, chlorophyll autofluorescence signal. To reduce the interference from chlorophyll autofluorescence in the green channel, we subtracted autofluorescence collected in the 680 nm channel from the green channel. The merged image was created using the green channel after subtraction and the original 680nm channel. White arrows indicate ROP4-swNG cortical enrichment at the onset of mitosis near the middle of a dividing cell in both cell 1 and cell 2. Cyan arrows indicate persistent ROP4-swNG cortical enrichment at the first branch site and the future second branch site. The dotted white lines in cell 1 and cell 2 were used to generate the kymographs labeled cell 1 and cell 2. Cyan line in the kymographs indicate the time period that ROP localized to the cell cortex before branch initiation; white horizontal line indicates the time point of the apical cell division. Scale bars for kymographs, 5 µm (x) and 2 hours (y). (B) Wild-type protonemal filaments expressing a nucleus-localized GFP:GUS fusion protein five minutes after infusion of Driselase into the PDMS imaging device. Images are maximum projections of confocal fluorescence and DIC z-stacks. Green, GFP signal; magenta, chlorophyll autofluorescence. DIC is shown in gray to indicate the cell outlines. White arrow indicates the apical cell. Black arrows indicate the position where the protoplasts emerge in subapical cells. Also see Supplemental Movie 11. Scale bar for all images, 20 µm.
A Fully Functional ROP Fluorescent Fusion Protein Reveals Roles for this GTPase in Subcellular and Tissue-level Patterning

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