Processes essential for *Physcomitrium patens* protonemal development require

distinct levels of total activity provided by functionally redundant PpROP

GTPases

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<u>Running title:</u>

ROP GTPase functions in *P. patens* development

<u>One-sentence summary:</u>

Knock-out, complementation, and overexpression experiments further defined PpROP

functions in *P. patens* development, and demonstrated their dependence on distinct

levels of total PpROP activity.

Abstract

RHO GTPases are key regulators of cellular and developmental processes in most
eukaryotic organisms. ROPs (RHO of plants) constitute a plant-specific RHO subfamily.
ROP families expanded and functionally diversified during the evolution of structurally
complex vascular plants, but generally contain few members in non-vascular plants with
ancient features. In vascular and non-vascular plants, ROP proteins are required for cell
polarization, directional cell expansion, and mitotic cell plate positioning. All these
processes are impaired by the disruption of PpROP activity in the non-vascular moss
Physcomitrium patens. The aim of the study presented here was to further characterize
PpROP functions during *P. patens* protonemal development by a) knocking out
individually or in all possible combinations each of the four Rp*ROP* genes, which encode
nearly identical proteins, b) complementing knock-out mutants with WT or mutant
PpROP isoforms, or with heterologous homologs, and c) performing overexpression
experiments. PpROPs were found to have additional previously unknown functions in
the regulation of cell proliferation, caulonema differentiation, and gametophore
formation. Furthermore, different cellular and developmental processes were shown to
require distinct levels of total PpROP activity, rather than individual PpROP isoforms.
Implications of the remarkable sequence conservation and functional integration within
the PpROP protein family are discussed.

Keywords *Physcomitrium patens*, RHO/ROP GTPases, GTP/GDP cycling, polarity,
directional cell expansion, cell plate positioning, cell proliferation, caulonema
differentiation, gametophore development, apical initial cells, plant evolution

Introduction
RHO (RAS homologous) family small GTPases (guanosine triphosphate hydrolases) are
expressed in most eukaryotic organisms (Boureux et al., 2007) and play key roles in the
regulation of essential cellular processes with important functions in the development of
multicellular tissues and organs (Etienne-Manneville and Hall, 2002; Kawano et al.,
2014). Most RHO GTPases are associated with the plasma membrane (PM) based on
posttranslational prenylation, interact with different effectors to trigger downstream
signaling specifically in the GTP-bound confirmation, and are inactive when bound to

GDP after GTP hydrolysis. While regulatory factors that control RHO activity by

promoting GDP for GTP exchange, by stimulating GTP hydrolysis or by modulating PM-

association are closely related in different organisms (Berken et al., 2005; Kost, 2010;

Hodge and Ridley, 2016), RHO-dependent downstream signaling appears to be much

less conserved. Different families of RHO effectors are controlling similar cellular

processes in animals and in plants (Heasman and Ridley, 2008; Yalovsky et al., 2008;

Müller, 2023; Ntefidou et al., 2023). Furthermore, RHO effectors playing key roles in the

regulation of important cellular processes in complex vascular plants are either entirely

missing (Eklund et al., 2010; Ou and Yi, 2022) or have completely different functions in

non-vascular plants with more ancient features (Ntefidou et al., 2023).

RHO functions in the control of developmentally relevant cellular processes including

polarization, directional growth, motility, and division have been extensively

characterized both in animals and in plants. However, the direct demonstration of RHO

functions in tissue and organ development based on the investigation of loss-of-function

mutants has been hampered by substantial redundancy within large families of RHO

proteins typically expressed in complex multicellular organisms and, possibly, by

essential roles of at least some of these proteins in the development of such organisms

(Mulvey and Dolan, 2023b).

ROP (RHO of Plants) functions have been most extensively studied in *Arabidopsis*

thaliana, a complex flowering plant with an elaborate vascular system, which expresses

11 AtROPs sharing 80-98 % amino acid sequence identity (Li et al., 1998; Winge et al.,

2000). The functional characterization of these proteins largely depended on the

investigation 1) of loss-of-function mutants, and 2) of effects of overexpressing either

wild type (WT) AtROPs, or mutant versions of these proteins locked in the GTP-bound

conformation (constitutively active) or displaying reduced nucleotide affinity (moderate

reduction: fast-cycling, strong reduction: dominant negative). Different AtROPs were

determined to play distinct but partially overlapping (Feiguelman et al., 2018) essential

roles in the control of the following cellular processes: 1) extremely polarized tip growth

displayed by pollen tubes (Li et al., 1999; Luo et al., 2017) and root hairs (Molendijk et

al., 2001; Denninger et al., 2019), 2) diffuse directional expansion of leaf epidermal

pavement cells (Fu et al., 2005; Lauster et al., 2022), 3) morphogenesis of single-celled

trichomes (Liu et al., 2023), and 4) mitotic cell plate positioning required for tissue

patterning in developing roots (Roszak et al., 2021).

Interestingly, Arabidopsis mutants disrupted in the expression of multiple At*ROP* genes

show severely aberrant root hair and/or epidermal leaf pavement cells morphogenesis,

but no substantial defects in tissue organization and organ development (Fu et al., 2005;

Ren et al., 2016). Similarly, the development of the liverwort *Marchantia polymorpha* is

not strongly affected by knocking out the single Mp*ROP* gene identified in this non-

vascular plant, even though defects in the formation of selected tissues and organs were

observed (Mulvey and Dolan, 2023b). The relatively weak phenotype of *M. polymorpha*

rop knock-out mutants obviously cannot be attributed to genetic redundancy. ROP

signaling therefore only appears to play a minor role in controlling the development in

this liverwort, which displays ancient features presumably similar to those of extinct

common ancestors of all land plants. By contrast, growth and development of the closely

related non-vascular moss *P. patens* are severely disrupted in knock-out or knock-down

mutants completely lacking or displaying minimal ROP activity (Burkart et al., 2015;

Cheng et al., 2020; Yi and Goshima, 2020; Bao et al., 2022). These mutants only form

tiny colonies of irregularly shaped cells, which are unable to directionally expand,

differentiate or form organized tissues. The phenotype of these mutants established that

P. patens development strictly depends on the total activity of the four nearly identical

PpROPs expressed in this moss, which share 99.5-100 % amino acid identity and

display sequence variability only outside of known functional domains at positions 148

and 149 (Eklund et al., 2010). However, currently available data concerning PpROP

functions leave several important questions unanswered: 1) does PpROP activity only

exert previously identified functions in the control or cell polarization, directional cell

expansion and mitotic cell plate positioning, or is this activity also required for other

cellular and developmental processes?, 2) are different processes controlled by

individual ROP isoforms or by the combined activity of multiple PpROPs?, 3) are the four

PpROPs functionally redundant or do they differentially contribute to total PpROP

activity?, and 4) which evolutionary mechanisms may account for the origin and

maintenance of the PpROP protein family with its exceptionally high amino acid

sequence conservation?

The development of haploid *P. pates* gametophytes, which are composed of protonemal

filaments and gametophores, is an ideal system to further characterize PpROP

functions. This process 1) dominates the *P. patens* life cycle, 2) relies on cell

polarization, tip growth, strictly controlled cell plate positioning, and additional cellular or

developmental processes, and 3) is highly amenable to genetic manipulation based on

homologous recombination and CRISPR/Cas (Schaefer and Zrÿd, 1997; Cove, 2005;

Collonnier et al., 2017; Rensing et al., 2020). Gametophyte development starts with the

germination of haploid spores, which results in the formation of branched filamentous

protonemata. These structures can also be induced to develop *in vitro* from isolated

protonemal protoplasts or explants, with protoplast-derived colonies more closely

recapitulating normal development (Grimsley et al., 1977; Kofuji and Hasebe, 2014). A

single apical initial cell at the tip of each protonemal filament expands by tip growth and

regularly divides transversely, resulting in filament elongation. The association of

different PpROPs with the PM of apical initial cells specifically at the tip, as well as

underneath the transversal cell wall separating these cells from subapical cells, is

consistent with essential functions of PpROP activity in tip growth and cell plate positing

(Le Bail et al., 2019; Cheng et al., 2020; Yi and Goshima, 2020). While young

protonemal filaments display chloronemal characteristics, the initial cells at their tips

undergo gradual caulonema differentiation, which is associated with 1) an increased rate

of tip growth and proliferation, 2) a reduction in chloroplast size and number, and 3) the

formation of oblique rather than perpendicular transversal cell walls (Jaeger and Moody,

2021). Interestingly, caulonema differentiation is stimulated by the phytohormone auxin

(Jang and Dolan, 2011) and has recently been demonstrated to be effectively blocked by

the RpROP effector PpRIC downstream of auxin-induced changes in gene expression

(Ntefidou et al., 2023). Filament branching as well as the formation of lateral buds

developing into gametophores are initiated by the asymmetric division of subapical

caulonemal cells. As this process is defective in mutants lacking 3 of the four PpROPs, it

also appears to be controlled by PpROP activity (Yi and Goshima, 2020). Mature

gametophores are composed of leafy shoots with leaf-like phyllids and filamentous

rhizoids, which are free of chloroplasts but otherwise closely resemble caulonemal

filaments and elongate based on the same mechanisms. Reproductive organs produced

at the tip of mature gametophores mediate sexual reproduction, which initiates

sporophyte development that corresponds to the short diploid phase of the life cycle. To

address open questions concerning PpROP functions during different phases of *P.*

patens gametophyte development, systematic knock-out, complementation and

overexpression experiments were performed.

Results

Individual Pp*ROP* genes are not essential for protonemal development

Previously reported quantitative polymerase chain reaction (RT-qPCR) and RNA-

sequencing data demonstrated similar expression levels of all four Pp*ROP* genes in

developing protonemata (Perroud et al., 2018, Ntefidou et al., 2023). RT-qPCR analyses

performed here essentially confirmed these observations, although Pp*ROP4* was found

to display about 2x higher transcript levels as compared to the other three genes

(Supplementary Fig. S1). Based on these data, all four PpROPs appear to contribute to

the control of protonemal growth and/or differentiation.

To systematically investigate PpROP functions in protonemal development, homologous

recombination (Schaefer et al., 1991) was employed to replace all Pp*ROP* genes

individually and in every possible combination by antibiotic resistance genes. Knockout

(KO) lines obtained were verified by PCR genotyping (Supplementary Figs. S2A and B,

S3 and S4). Higher order mutants were generated sequentially, starting with *rop1*, *rop2*,

rop3, and *rop4* single KO lines (Supplementary Fig. S2C, upper panel). To enable

comparative quantitative characterization of KO phenotypes, protonemata regenerated

from isolated protoplasts were investigated at defined developmental stages. At least

two independent lines per genotype were generally characterized, except for the *rop2*,

rop4, r*op4/1* and *rop2/4/1* genotypes, which were only obtained once. Independently

generated genotypically identical lines in all cases displayed indistinguishable

phenotypes.

None of the four single *rop* KO mutants (*rop*^{1xKO}) displayed detectable defects in a) the

length of fully expanded subapical chloronemal or caulonemal cells (Supplementary Fig.

S5A), b) the morphology of the tips of chloronemal or caulonemal filaments

(Supplementary Fig. S5B), c) the rate of caulonema differentiation (Supplementary Fig.

S5C), which is enhanced in a KO mutant lacking the PpROP effector PpRIC (*ric-1*^{KO},

Ntefidou et al., 2023), or d) the size or morphology of 5-day-old protonemata or of 5
week-old colonies (Supplementary Fig. S5D-F). These observations establish that each
individual *ROP* gene is dispensable for cell expansion and differentiation during
protonemal development.

Mutants lacking any combination of two Pp*ROP* genes display enhanced
caulonema differentiation

The six rop^{2xKO} mutants lacking two Pp*ROP* genes in all possible combinations did not
show detectable defects in cell expansion or morphology in 5-day-old protonemata,
except for an apparent minor decrease in the size of *rop4/1* protonemata (Fig. 1A, B, D
and F). However, at the same developmental stage caulonema differentiation was
substantially enhanced to a similar extent in all rop^{2xKO} mutants (Fig. 1C), establishing
an essential function of PpROP activity in the inhibition of this process. Caulonema
differentiation was proposed to be triggered when elongating apical chloronemal cells
reach a threshold length (Jang and Dolan 2011). Since the length of chloronemal cells
was not affected in rop^{2xKO} mutants (Fig. 1A), enhanced caulonema differentiation
displayed by these mutants is not a secondary effect of altered cell expansion, but
appears to be a direct consequence of reduced PpROP activity.

As none of the rop^{1xKO} mutants showed enhanced caulonema differentiation
(Supplementary Fig. S5C), any combination of three Pp*ROP* genes evidently is sufficient
for the inhibition of this process in normally developing protonemata, which has
previously been shown to also require the PpROP effector PpRIC (Ntefidou et al., 2023).
PpRIC was demonstrated to inhibit caulonema differentiation downstream of gene
expression controlled by indole-3-acetic acid (IAA), an auxin-family phytohormone with a
key role in the stimulation of this process (Jaeger and Moody, 2021). To further
investigate whether PpROP activity and PpRIC act together in the same pathway to
inhibit caulonema differentiation as proposed by Ntefidou et al. (2023), IAA levels, a
marker for auxin transport and auxin dependent-gene expression were analyzed in
rop^{2xKO} mutants. Like $ric\text{-}1^{KO}$ mutants (Ntefidou et al., 2023), all rop^{2xKO} mutants were
found a) to contain wild-type IAA levels (Supplementary Fig. S6A), b) to display normal
accumulation of the key auxin transporter PpPINA (PIN-FORMED A auxin efflux carrier;
Viaene et al., 2014) specifically in the PM at the tip of apical protonemal cells

(Supplementary Fig. S6B), and c) to express at normal levels different auxin-responsive

genes (Supplementary Fig. S6C), which are required for auxin-induced caulonema

differentiation (Pp*RSL1*; *ROOTHAIR DEFECTIVE SIX-LIKE1*; Jang et al., 2011), or have

essential functions in auxin biosynthesis (Pp*SHI1*; *SHORT INTERNODES1*; Eklund et

al., 2010b), transport (Pp*PINA*) or signaling (Pp*AUX1*; *AUXIN RESISTANT 1*; Lavy et

al., 2016). These observations establish that PpROP activity, like PpRIC, inhibits

caulonema differentiation without affecting auxin-controlled gene expression, supporting

the notion that PpROP activity regulates this process via PpRIC stimulation.

Consistent with a considerably higher growth rate of caulonemal as compared to

chloronemal filaments (Cove and Knight, 1993), enhanced caulonema differentiation

substantially enlarged the size of 5-week-old *ric-1*^{KO} colonies (Ntefidou et al., 2023). By

contrast, despite enhanced caulonema differentiation, the size of 5-week-old *rop*^{2xKO}

colonies was significantly reduced (Fig. 1E and G), although as discussed above, 5-day-

old *rop*^{2xKO} protonemata did not display substantial growth defects (Fig. 1A, B, D and F).

Based on these observations, PpRIC appears to be exclusively required for the

inhibition of caulonema differentiation, whereas RpROP activity, as previously

demonstrated, also promotes cell and protonemata expansion, evidently by stimulating

other effectors. However, it is important to note that *rop*^{2xKO} protonemata displayed

minimal growth defects, which only became apparent late during development and did

not prevent the detection of enhanced caulonema differentiation at an earlier stage.

Cell and protonemal expansion are severely disrupted in mutants lacking any combination of three Pp*ROP* genes

Consistent with a previously reported phenotypic analysis of *P. patens rop1/2/3* and

rop2/3/4 triple KO mutants (Yi and Goshima, 2020), 5-day-old protonemata of each of

the four *rop*^{3xKO} mutants lacking three Pp*ROP* genes in all possible combinations

displayed severe growth defects (Fig. 2A, B, D and G). Already at this early

developmental stage, the length of apical and subapical chloronemal and caulonemal

cells (Fig. 2A and B), as well as the size of protonemata (Fig. 2D and G), were

substantially decreased. Interestingly, at the same developmental stage and by contrast

to *rop*^{2xKO} mutants, *rop*^{3xKO} mutants did not display enhanced caulonema differentiation

(Fig. 2C). As caulonema differentiation seems to depend on a cell length threshold (Jang

and Dolan, 2011), reduced cell expansion appears to inhibit this process in rop^{3xKO}

protonemata.

The analysis of 5-week-old rop^{3xKO} colonies revealed even more pronounced growth

defects (Fig. 2E and H). Interestingly and consistent with previously reported

observations (Yi and Goshima, 2020), at this developmental stage rop^{3xKO} colonies

formed gametophores with small but morphologically essentially normal leafy shoots

and even more severely stunted rhizoids (Fig. 2F, H and I), which like protonemata are

composed of filaments that elongate based on apical tip growth. The phenotypic

characterization of rop^{3xKO} protonemata and gametophores therefore not only confirms

essential functions of PpROP activity in the control of tip growth but also suggests less

important roles of this activity in other forms of directional cell expansion, which are

required for leafy shoot development. Whereas tip growth is strongly reduced in all

rop^{3xKO} mutants (Fig. 2), this process is only marginally affected in all rop^{2xKO} mutants

(Fig. 1). The expression of any combination of two PpROPs therefore appears to be

sufficient to support nearly normal tip growth.

All four PpROPs are equally capable of promoting tip growth

Amino acid sequence identities of at least 99.5 % within the PpROP protein family,

together with highly similar phenotypes of all rop^{2xKO} (Fig. 1) and rop^{3xKO} (Fig. 2) mutants

revealed by quantitative characterization, suggest essentially equivalent functions in all

four PpROPs in developing protonemata. However, cell and protonema expansion was

reduced to a remarkably variable extent in rop^{3xKO} mutants expressing distinct single

PpROP family members (Fig. 2B, D and E), indicating that these proteins may differ in

their ability to promote tip growth. Alternatively, the observed phenotypic variability of

rop^{3xKO} mutants may be caused a) by differential up- or down-regulation of the

expression of the remaining PpROP gene after the disruption of the other members of

the PpROP gene family, b) by different genetic backgrounds resulting from multiple

rounds of gene replacement based on homologous recombination, and/or c) by the

expression of variable sets of selectable marker genes resulting from this procedure.

RT-qPCR analysis established that in all four rop^{3xKO} mutants the remaining PpROP

gene was expressed at the same level as the corresponding gene in the wild type

background (Fig. 3A). This demonstrates that the phenotypic variability displayed by
rop^{3xKO} mutants is not caused by differential feed-back control of transcript levels within
the Pp*ROP* gene family. To distinguish between the other possible causes of phenotypic
variability displayed by rop^{3xKO} mutants, the activity of all PpROP family members was
directly compared by individually expressing each of them under the control of the same
promoter in an identical genetic background. To this end, based on homologous
recombination the coding sequence (exons and introns) and 3′ untranslated region
(UTR) of the endogenous Pp*ROP1* gene in the *rop3/2/4* background was replaced by
CDS (coding sequence) fragments encoding PpROP1, PpROP2, or PpROP3, which
were attached to the Pp*ROP1*, Pp*ROP2*, or Pp*ROP3* 3′ UTR, respectively
(Supplementary Fig. S2A and C: lower panel). As PpROP1 and PpROP4 share identical
amino acid sequences, the Pp*ROP4* CDS was not included in this analysis. Resulting
rop^{4xKO}/*ROP1*^{pro}:*ROPX* lines (at least two per genotype) were confirmed by PCR
genotyping (Supplementary Fig. S2B) and in essence represent rop^{4xKO} mutants sharing
an identical genetic background, which are complemented by the expression of different
single PpROPs under the control of the endogenous Pp*ROP1* promoter. RT-qPCR
analysis confirmed that each of the *ROP1*^{pro}:*ROPX* genes was expressed in the rop^{4xKO}
background at a similar level as the endogenous Pp*ROP1* gene in the *rop3/2/4* mutant
or in WT protonema (Fig. 3B). Size and morphology of 5-day-old protonemata as well as
of 5-week-old colonies formed by all rop^{4xKO}/*ROP1*^{pro}:*ROPX* lines were found to be
indistinguishable (Fig. 3C-E), establishing that all four 4 PpROPs are equally capable of
promoting tip growth.

Disruption of all four Pp*ROP* genes completely abolishes polarized cell expansion
and affects cell division
Two independent rop^{4xKO} lines were generated (Supplementary Fig. S2C: upper panel)
by knocking out based on homologous recombination either Pp*ROP1* in the *rop3/2/4*
background (*rop3/2/4/1*; Supplementary Fig. S2A and B) or Pp*ROP2* in the *rop3/4/1*
background (*rop3/4/1/2*; Supplementary Fig. S3A and B). Both rop^{4xKO} lines displayed
the same phenotype (Fig. 4A) as previously described *P. patens* mutants, in which the
expression of all 4 *PpROP* genes was disrupted either by RNAi/microRNA-mediated
downregulation (Burkart et al., 2015; Bao et al., 2022), by CRISPR/Cas9 (clustered

regularly interspaced short palindromic repeats/CRISPR-associated nuclease 9)-
mediated KO (Cheng et al., 2020), or by a combination of these approaches (Yi and
Goshima, 2020). Mutants entirely or nearly completely lacking PpROP activity form tiny
colonies of irregularly shaped cells (Fig. 4A). By contrast to cells containing at least one
functional PpROP gene (*rop*[3xKO]), protonemal *rop*[4xKO] cells were unable a) to undergo
normal polarization, b) to maintain parallel orientation of cell division planes required for
the development of linear filaments, or c) to directionally expand (Fig. 4A and B). When
cultured for prolonged periods of time, *rop*[4xKO] colonies continued to slowly grow based
on cell division but failed to differentiate and did not develop gametophores.
Interestingly, *rop*[4xKO] lines essentially phenocopy *P. patens* KO mutants lacking
geranylgeranyltransferase type I activity (Thole et al., 2014), suggesting that PpROPs
require prenylation by this activity to be functional (Bao et al., 2022).

In addition to the defects described in the previous paragraph, quadruple Pp*ROP* RNAi
knock-down mutants were demonstrated to also display reduced cell adhesion and
altered cell wall composition (Burkart et al., 2015; Bao et al., 2022). Consistent with
these observations, protoplasts could not be isolated from colonies formed by the two
different *rop*[4xKO] mutants described here, hampering the comparative quantitative
phenotypic characterization of these mutants. To circumvent this issue, conditionally
complemented *rop*[4xKO] lines were generated. Based on homologous recombination the
coding sequence (exons and introns) and 3′ UTR of the Pp*ROP3* gene in the *rop2/4/1*
background was replaced by a CDS fragment encoding PpROP2, which was fused at 5'-
end to an estradiol-inducible lexa/35S promoter (Kubo et al., 2013) and at the other end
to the Pp*ROP2* 3′ UTR (Supplementary Figs. S2C: lower panel; and S3C). The two
independent *rop*[4xKO]/*ind*[pro]:*ROP2* lines obtained based on this procedure were confirmed
by PCR genotyping (Supplementary Fig. S3D). In the absence of estradiol, these lines
produced Pp*ROP2* transcripts at substantially lower levels as compared to the *rop3/4/1*
mutant (Fig. 4C), and displayed a typical *rop*[4xKO] phenotype after 5 days and after 5
weeks in culture (Fig. 4D). Adding 10^{-3} µM estradiol to the culture medium resulted in
partial complementation of the *rop*[4xKO] phenotype, which enabled protoplast isolation.

Quantitative comparison of protoplast-derived 5-day-old protonemata growing on
estradiol-free culture medium confirmed that *rop*[4xKO]/*ind*[pro]:*ROP2* colonies displaying a

rop[4xKO] phenotype were significantly smaller than *rop3/4/1* colonies (Fig. 4E), which

showed the strongest growth defects of all *rop*[3xKO] mutants when compared to WT (Fig.

2D and E). Remarkably, counting DAPI (4′,6-diamidino-2-phenylindole)-labeled nuclei in

protoplast-derived protonemata grown for 4 days in the absence of estradiol (Fig. 4F)

established that the observed stepwise reduction of the size of WT, *rop3/4/1* and

rop[4xKO]/*ind*[pro]:*ROP2* (*rop*[4xKO] phenotype) protonemata (Fig. 4E) is a consequence not

only of inhibited cell expansion but also of a similar stepwise decrease in the rate of cell

division (Fig. 4G). The cell division rate of apical protonemal cells is enhanced by

caulonema differentiation (Cove and Knight, 1993; Jang and Dolan, 2011) and possibly

by rapid cell expansion (Fantes and Nurse, 1977; Jones et al., 2017). The complete

disruption of both these processes in *rop*[4xKO] mutants (Fig. 4) and the partial inhibition of

cell expansion in *rop*[3xKO] mutants (Fig. 2), therefore appear likely to be partially

responsible for the reduced cell proliferation displayed by these mutants. However, like

related mammalian RHO GTPases (Coleman et al., 2004), PpROPs may also directly

target cell cycle regulation to enhance the rate of cell division, in addition to promoting

cell expansion and differentiation.

Comparing the phenotypes of *rop*[4xKO] mutants and of the four *rop*[3xKO] mutants

expressing distinct individual PpROPs (Figs. 2-4) establishes that each of these proteins

alone is capable of inducing not only normal polarization and directional expansion

(albeit at the reduced rate) of protonemal cells, but also parallel cell division plane

positioning, which is required for the development of linear filaments. Consistent with an

important role of PpROP activity in cell division plane positioning, defects in this process

were also observed a) in asymmetrically dividing cells in Arabidopsis roots

overexpressing constitutively active mutant AtROP9 (Roszak et al., 2021) or in maize

leaves lacking functional Zm*ROP2* and Zm*ROP9* genes (Humphries et al., 2011), b)

during branch formation in *P. patens rop*[3xKO] protonemata (Yi and Goshima, 2020), and

c) in different tissues of the closely related liverwort *Marchantia polymorpha* after the

disruption of the single Mp*ROP* gene expressed in this organism (Mulvey and Dolan,

2023b). Interestingly, quantitative phenotypic characterization of *rop*[3xKO] and *rop*[4xKO]

mutants further demonstrated that PpROP activity directly and/or indirectly promotes the

rate of cell division in growing protonemata. In summary, based on the investigation of

these mutants PpROP activity was determined to control protonemal morphogenesis by
promoting cell polarization, parallel cell division plane positioning, cell proliferation, and
directional cell expansion.

Estradiol-induced Pp*ROP2* expression at increasing levels progressively
complements different defects displayed by *rop*^{4xKO} mutants

Data generated by the quantitative phenotypic characterization of *rop*^{KO} mutants (Figs.
1,2 and 4), and by the investigation of the ability of different PpROPs to complement
rop^{4xKO} phenotypes (Fig. 3), strongly suggests that a) the four nearly identical PpROPs
are functionally redundant, and b) increasing levels of total PpROP activity in apical
protonemal cells are required for the normal polarization, division, expansion and
differentiation of these cells (in this order). To further support these findings, the
phenotypes of *rop*^{4xKO}/*ind*^{pro}:*ROP2* protonema were investigated, in which Pp*ROP2*
expression was gradually induced by increasing estradiol concentrations in the culture
medium. Estradiol concentrations eliciting striking phenotypic alterations were selected
for this analysis (Fig. 5A). Control experiments confirmed that estradiol at these
concentrations did not affect the development of WT protonemata (Supplementary Fig.
S7).

At concentrations of up to $4x10^{-4}$ µM, estradiol-induced Pp*ROP2* expression remained
much lower as compared to the WT expression level of this gene (Fig. 5B), which also
corresponds to the level of total Pp*ROP* gene expression in *rop3/4/1* and in other *rop*^{3xKO}
mutants (Fig. 3A, Supplementary Fig. S1). A linear correlation between estradiol
concentrations of up to $4x10^{-4}$ µM and resulting minimal levels of Pp*ROP2* expression,
which were near the detection limit, could not be established based on RT-qPCR
analysis (Fig. 5B). Pp*ROP2* expression induced by $9x10^{-4}$ µM estradiol was about 2.3x
stronger than in WT protonemata (Fig. 5B) and thus reached a level comparable to total
Pp*ROP* gene expression in *rop*^{2xKO} mutants (2-3x WT Pp*ROP2* expression level;
Supplementary Fig. S1). Unfortunately, higher estradiol concentrations failed to further
enhance Pp*ROP2* expression to levels comparable to total Pp*ROP* gene expression in
normally developing *rop*^{1xKO} or WT protonemata (3-4x or 5x WT Pp*ROP2* expression
level, respectively; Supplementary Fig. S1).

As already shown above (Fig. 4D), in the absence of estradiol rop^{4xKO}/ind^{pro}:$ROP2$

protonema displayed a typical rop^{4xKO} phenotype after 5 days and after several weeks in

culture (Fig. 5). Remarkably, induction of Pp$ROP2$ expression at the lowest level by

$2x10^{-5}$ µM estradiol partially restored cell polarization and parallel cell division plane

positioning required for filament formation (Fig. 5A), without substantially promoting

directional cell expansion (Fig. 5C and E, Supplementary Table S1) or supporting

caulonema differentiation (Fig. 5D). These observations demonstrate that cell

polarization and parallel cell division plane positioning can be uncoupled from directional

cell expansion, and that these processes depend on different levels of total PpROP

activity.

On solid culture medium containing $2x10^{-4}$ µM estradiol, at low frequency chloronemal

filaments were formed, which elongated based on directional cell expansion (Fig. 5A,

Supplementary Table S1). However, this process was not fully restored, as subapical

cells in these filaments were much shorter than corresponding WT cells (Fig. 5C), and

colony growth remained strongly restricted (Fig. 5A and E). Caulonema differentiation

(Fig. 5A and D) or gametophore formation (Fig. 5A) was not observed under these

conditions. Interestingly, colonies growing for 4 weeks in liquid medium containing

estradiol at the same concentration, and displaying an otherwise indistinguishable

phenotype, frequently developed small gametophore-like structures, which contained

two to four rudimentary phyllids, but failed to form stems or rhizoids (Fig. 5A). This

finding is consistent with previous reports demonstrating that liquid culture can promote

gametophore formation (Reski and Abel, 1985). As similar gametophore-like structures

were never formed by rop^{4xKO}/ind^{pro}:$ROP2$ colonies growing in liquid medium containing

estradiol at lower concentrations, PpROP activity at minimal levels appears to be able to

induce the formation of gametophore-like structures even in the absence of caulonema

differentiation. Furthermore, PpROP activity at these levels evidently is sufficient for the

development of rudimentary phyllids.

Pp$ROP2$ expression induced by $4x10^{-4}$ µM estradiol further promoted directional cell

expansion (Fig. 5A and C; Supplementary Table S1) and partially rescued caulonema

differentiation (Fig. 5A and D). This led to a substantial increase in the size of 4-week-old

colonies (Fig. 5A and E) and was accompanied by the frequent development of regular

gametophores with a reduced size and severely stunted rhizoids on solid culture
medium (Fig. 5A). However, the length of subapical protonemal cells, the rate of
caulonema differentiation, as well as the size of 5-days-old and 4-weeks-old colonies
remained significantly decreased. Interestingly, essentially the same developmental
defects are shown by rop^{3xKO} mutants (Fig. 2), although the phenotype of these mutants
appears to be somewhat weaker, as they display caulonema differentiation at WT rates,
which are significantly reduced only in comparison to rop^{2xKO} mutants.

Finally, rop^{4xKO}/ind^{pro}:*ROP2* protonemata growing in medium containing 9×10^{-4} μM
estradiol essentially phenocopied rop^{2xKO} mutants (Figs. 1 and 5, Supplementary Table
S1), which as discussed above exhibit similar levels of total Pp*ROP* gene expression. As
compared to WT protonemata, rop^{4xKO}/ind^{pro}:*ROP2* protonemata expressing Pp*ROP2* at
this level of induction displayed significantly enhanced caulonema differentiation (Fig.
5D), while subapical cell length in 5-day-old protonemata was not detectably affected
(Fig. 5C), and colony size after 5 days or after 4 weeks in culture as well as
gametophore growth were only weakly reduced (Figs. 5A and E).

Data presented in this section demonstrate that PpROP2, a single member of the
PpROP family, can effectively complement different severe defects displayed by rop^{4xKO}
mutants. Partially interdependent developmental processes disrupted in these mutants
were progressively restored by increasing levels of estradiol-induced Pp*ROP2*
expression, indicating that these processes require distinct levels of total PpROP
activity, rather than specific individual ROPs. Listed in the order of their increasing
requirement for total PpROP activity, the following processes were restored by estradiol-
induced PpROP2 expression: a) cell polarization and parallel cell division plane
positioning required for filament formation, b) directional cell expansion, and c)
development of gametophore-like structures or small gametophores, and d) caulonema
differentiation.

Pp*ROP* overexpression inhibits caulonema differentiation and depolarizes
directional cell expansion
To investigate effects of PpROP overexpression, based on homologous recombination a
CDS fragment encoding PpROP1, which was fused at the 5'-end to the estradiol-

inducible lexa/35S promoter and at the other end to the Pp*ROP1* 3′ UTR, was

introduced into a neutral region (Schaefer et al., 1991) of the WT genome

(Supplementary Fig. S2D). One of the resulting WT/*ind*[pro]:*ROP1* lines, which displayed

massive estradiol-inducible Pp*ROP1* overexpression, was confirmed by PCR

genotyping (Supplemental Fig. S2E) and selected for phenotypic characterization. In the

absence of estradiol, protoplast-derived protonemata of this line expressed Pp*ROP1* at

WT level (Fig. 6A) and displayed no developmental defects (Fig. 6B-H). Growing these

protonemata on solid medium containing 10^{-3} µM estradiol resulted in Rp*ROP1*

expression at a more than 150x higher level (Fig. 6A), which roughly corresponds to a

30x increase in total Pp*ROP* gene expression as compared to WT protonemata (total

Pp*ROP* gene expression ca. 5x stronger than Pp*ROP1* expression; Supplementary Fig.

S1). Control experiments showed that estradiol at this concentration did not affect

Pp*ROP1* expression or developmental processes in WT protonemata (Supplementary

Fig. S7).

As discussed above, PpROP activity was concluded to directly inhibit caulonema

differentiation based on the observation that this process is enhanced in otherwise

essentially normally developing rop^{2xKO} protonemata (Fig. 1). Further supporting this

conclusion, Pp*ROP1* overexpression effectively disrupted caulonema differentiation (Fig.

6B). In angiosperm pollen tubes and root hairs excess ROP activity strongly depolarizes

tip growth and results in massive apical ballooning (Chen et al., 2003; Gu et al., 2003a;

Klahre and Kost, 2006; Kost, 2008). Overexpression of a fluorescent PpROP2 fusion

proteins was reported to have similar but comparably weaker effects on directional

expansion at the tip of *P. patens* protonemata (Ito et al., 2014). Quantitative analysis of

protoplast-derived WT/*ind*[pro]:*ROP1* protonemata revealed that high-level Pp*ROP1*

overexpression strongly reduced the length and enhanced the width of subapical cells in

chloronemal filaments (Fig. 6C-E), which indicates massive depolarization of tip growth

displayed by adjacent apical cells. Interestingly, PpPOP1 overexpression caused the

apical dome of these cells to oscillate at irregular intervals between a wide flattened and

a narrow pointed morphology (Fig. 6E). Together, inhibited caulonema differentiation and

defective cell expansion strongly reduced the size of Pp*ROP1*-overexpressing 5-day-old

protonemata and of 4-week-old colonies (Fig. 6F-H). Remarkably, WT/*ind*[pro]:*ROP1*

protonemata never developed gametophores when growing on solid or in liquid medium

containing 10^{-3} µM estradiol, demonstrating that PpPOP1 overexpression effectively

blocked the formation of these structures (Fig. 6H).

In summary, consistent with and further defining PpROP functions identified by the

phenotypic characterization of *rop*[KO] mutants, PpROP1 overexpression prevented

caulonema differentiation, effectively depolarized cell expansion at the tip of protonemal

filaments, strongly reduced protonemal growth, and completely blocked gametophore

formation. Overexpression of the PpROP effector PpRIC (ca. 40x WT Pp*RIC* expression

level) also strongly inhibited caulonema differentiation and gametophore formation, but

did not depolarize directional cell expansion and, consequently, only weakly reduced

protonemal growth (Ntefidou et al., 2023). These observations strongly suggest that

PpROP inhibits caulonema differentiation via PpRIC activation, but interacts with other

effectors to control directional cell expansion. As discussed above, this conclusion is

further supported by the increase or decrease in colony size displayed by *ric-1*[KO] and

rop2x[KO] mutants, respectively. PpROP and PpRIC may also act together in a common

pathway to directly inhibit gametophore formation, as this process is completely blocked

by the overexpression of either of these two proteins. However, chloronemal filaments

were proposed to lack competence for gametophore formation during normal

protonemal development (Cove and Knight, 1993; Schumaker and Dietrich, 1997; Brun

et al., 2003; Harrison et al., 2009), indicating that PpROP or PpRIC overexpression may

indirectly block gametophore formation by inhibiting caulonema differentiation.

Consistent with this interpretation, increasing ROP activity progressively stimulates the

formation of gametophore-like structures and the development of gametophores (Figs. 2

and 5), suggesting that ROP activity promotes rather than inhibits these processes.

PpROP functions in fundamental developmental processes depend on GDP/GTP
cycling

Highly conserved amino acid residues can be mutated to alter the intrinsic rate of GDP/

GTP cycling displayed by RHO GTPases (Aspenström, 2019). These proteins are

locked in the GTP-bound state by mutations corresponding to the Q61L exchange in H-

RAS, which disrupt GTPase activity (GTP-locked). By contrast, mutations equivalent to

the F28L exchange in H-RAS reduce nucleotide-binding affinity and enhance intrinsic

GDP/GTP exchange (fast-cycling). Fast-cycling RHO mutants are preferentially GTP-

bound at high GTP/GDP concentration ratios typically observed in the cytoplasm of

eukaryotic cells. Like GTP-locked mutants, fast-cycling mutants are therefore

considered to be constitutively active. To assess the importance of GDP/GTP cycling for

PpROP functions in protonemal development, based on homologous recombination the

coding sequence (exons and introns) and 3′ UTR of the endogenous Pp*ROP1* gene in

the *rop3/2/4* background was replaced by CDS fragments encoding GTP-locked

PpROP1^{Q64L} or fast-cycling PpROP1^{F31L}, which were attached to the Pp*ROP1* 3′ UTR

(Supplementary Fig. S2A and C: lower panel). Resulting *rop*^{4xKO}/*ROP1*^{pro}:*ROP1*^{Q64L} and

rop^{4xKO}/*ROP1*^{pro}:*ROP1*^{F31L} lines were confirmed by PCR genotyping (Supplementary

Fig. S2B). Based on RT-qPCR analysis of 1-week-old protonemata, lines expressing

mutated Pp*ROP1* genes at the same level as Pp*ROP1* expression in WT plants and in

rop3/2/4 mutants were identified (Fig. 7A) and selected for phenotypic characterization

(Fig. 7B-D).

Even after prolonged culture on solid medium, *rop*^{4xKO}/*ROP1*^{pro}:*ROP1*^{Q64L} colonies

displayed a typical *rop*^{4xKO} phenotype (Fig. 7B). As this phenotype includes cell wall

defects preventing protoplast isolation, *rop*^{4xKO}/*ROP1*^{pro}:*ROP1*^{Q64L} colonies shown were

grown from tissues explants. This remarkable observation established that GDP/GTP

cycling is strictly required for PpROP functions in fundamental developmental

processes, including cell polarization and parallel division plane positioning required for

filament formation. It remains to be determined whether GDP/GTP exchange is also

essential for ROP functions in directional cell expansion and other processes, which

appear to directly or indirectly depend on cell polarization and/or parallel division plane

positioning. Interestingly, like *rop*^{4xKO}/*ind*^{pro}:*ROP2* colonies in the presence of estradiol at

low concentration ($2x10^{-4}$ μM; Fig. 5A), *rop*^{4xKO}/*ROP1*^{pro}:*ROP1*^{Q64L} colonies frequently

formed gametophore-like structures exclusively when grown in liquid medium (Fig. 7C).

The induction of gametophore formation in the absence of caulonema differentiation, as

well as the promotion of rudimentary phyllid development by low-level PpROP activity,

therefore does not require GDP/GTP cycling.

Protoplast-derived 5-day-old and 5-week-old *rop*^{4xKO}/*ROP1*^{pro}:*ROP1*^{F31L} and *rop3/2/4*

protonemata displayed indistinguishable phenotypes (Fig. 7B). As compared to WT

protonemata, colony size was similarly reduced (Fig. 7D), caulonema differentiation was not significantly affected (Fig. 7E), and gametophores with small but morphologically essentially normal leafy shoots and severely stunted rhizoids were formed (Fig. 7C). These data establish that by contrast to GTP-locked PpROP1^{Q64L}, which is severely functionally impaired, fast-cycling PpROP1^{F31L} displays essentially the same ability as WT PpROP1 to promote protonemal development. However, it remains to be demonstrated that PpROP1^{F31L} is also capable of inhibiting caulonema differentiation when expressed at endogenous level in a rop^{2xKO} mutant, or upon overexpression.

Overexpression of heterologous RHO GTPases in the rop^{4xKO} background suggests limited evolutionary conservation of PpROP downstream signaling

To test the ability of heterologous RHO GTPases to complement total loss of PpROP activity, AtROP7, the closest Arabidopsis PpROP homologue (Eklund et al., 2010), and human HsRHOA, one of the most extensively characterized RHO GTPases (Bishop and Hall, 2000), were overexpressed in the rop^{4xKO} background. To this end, based on homologous recombination, the coding sequence (exons and introns) of the endogenous Pp*ROP4* gene in the *rop3/1/2* background was replaced by CDS fragments encoding AtROP7 or HsRHOA, which were fused at 5'-end to the estradiol-inducible lexa/35S promoter (Supplementary Fig. S4A). Resulting rop^{4xKO}/*ind*^{pro}:At*ROP7* and rop^{4xKO}/*ind*^{pro}:Hs*RHOA* lines were confirmed by PCR genotyping (Supplementary Fig. S4B). Based on RT-qPCR analysis of 1-week-old protonemata (Fig. 8A), lines displaying maximal At*ROP7* or Hs*RHOA* transcript levels upon estradiol-induction were selected for phenotypic characterization (Fig. 8B and C). Maximal At*ROP7* and Hs*RHOA* transcript levels obtained were about 40x or more than 500x, respectively, higher than Pp*ROP4* transcript levels in WT plants and in *rop3/1/2* mutants (Fig. 8A), which roughly corresponds to 16x or more than 200x higher transcript levels as compared to total Pp*ROP* gene expression in WT protonemata (ca. 2,5x stronger than Pp*ROP4* expression; Supplementary Fig. S1). Control experiments showed that estradiol at concentrations of 10^{-3} µM or 1 µM, which maximally induced At*ROP7* or Hs*RHOA* expression, respectively, did not affect developmental processes in WT protonemata (Supplementary Fig. S7).

Remarkably, massive At*ROP7* or Hs*RHOA* over-expression complemented the *rop*^{4xKO}

phenotype to a similar extent (Fig. 8B and C) as estradiol-induced Pp*ROP2* expression

at minimal level ($2x10^{-5}$ µM; Fig. 5). Cell polarization and parallel cell division plane

positioning required for filament formation were restored, whereas directional cell

expansion was not substantially promoted and caulonema differentiation was never

induced (Fig. 8B). Furthermore, even on solid culture medium, small gametophore-like

structures occasionally developed, which contained a few rudimentary phyllids, but

unlike regular WT or *rop*^{3xKO} gametophores failed to form stems or rhizoids (Fig. 8C).

These observations establish that massive overexpression of heterologous flowering

plant and mammalian RHO GTPases can restore fundamental developmental

processes in *rop*^{4xKO} mutants, which require minimal levels of total PpROP activity.

Consequently, PpROP downstream signaling controlling these processes appears to be

evolutionary conserved at least to some extent. Supporting this conclusion, in *P. patens*

KO mutants lacking geranylgeranyltransferase type I activity, which as discussed display

a *rop*^{4xKO} phenotype, cell polarization and parallel cell division plane positioning could be

restored by a YFP-AtROP1 fusion protein mutated to enable prenylation by

farnesyltransferases. However, when tested using the same approach, human HsRAC1

or HsKRAS4b did not show a similar ability to complement loss of PpROP activity (Bao

et al., 2022).

Discussion

PpROP functions in protonemal development were systematically investigated based on

quantitative phenotypic characterization a) of KO mutants lacking single Pp*ROP* genes

or multiple such genes in all possible combinations, b) of complemented *rop*^{4xKO} lines

expressing WT PpROPs, constitutively active PpROP1 mutants or heterologous RHO

GTPases either at endogenous or different estradiol-titrated levels, and c) of lines

displaying PpROP1 overexpression in the WT background. The results of these

analyses firmly establish a) that the four nearly identical PpROPs, which are co-

expressed at similar levels in protonemata, are functionally redundant, and b) that

different developmental processes depend on distinct levels of total PpROP activity,

rather than on specific individual ROPs (Fig. 9).

PpROP activity induces cell polarization and parallel division plain positioning at minimal levels, and increasingly promotes tip growth when further enhanced

Low levels of total PpROP activity induce cell polarization and parallel cell division plane positioning, which enables filament formation, but are insufficient for the promotion of directional cell expansion. Tip growth of apical protonemal cells is induced at somewhat higher total PpROP activity and is further promoted by increasing levels of this activity until it reaches WT rates in rop^{1xKO} mutants (Fig. 9). High-level PpROP overexpression massively depolarizes tip growth, similar to what has been observed in vegetative pollen tube cells and in root hairs (Chen et al., 2003; Gu et al., 2003a; Klahre and Kost, 2006; Kost, 2008). However, unlike these other cell types apical protonemal cells overexpressing PpROP activity do not simply swell at the tip, but display complex growth behavior that warrants further investigation (Fig. 6E). Despite these somewhat different overexpression effects, ROP functions in the control of tip growth appear to be essentially conserved from mosses to flowering plants. Yeast and mammalian RHO GTPases also have well-characterized functions in the regulation of directional cell expansion and related processes (Hall and Lalli, 2010; Ou and Yi, 2022).

PpROP activity at WT and somewhat lower levels blocks caulonema differentiation via PpRIC stimulation

As caulonema differentiation is substantially enhanced both in rop^{2xKO} and in ric-1^{KO} mutants, total PpROP activity at levels reached in rop^{1xKO} and in WT protonemata appears to inhibit caulonema differentiation by stimulating the effector protein PpRIC (Fig. 9; Ntefidou et al., 2023). This conclusion is further supported by the recent demonstration that inhibition of PpROP activity by upstream regulators of the PpGAP (GTPase activating proteins) and PpREN (ROP Enhancer) families also promotes caulonema differentiation (Ruan et al., 2023). Consistent with these findings, caulonema differentiation is almost completely blocked by PpROP1 (Fig. 9) or PpRIC (Ntefidou et al., 2023) overexpression. However, as discussed above, apical chloronemal cells were proposed to undergo caulonema differentiation after reaching a threshold length (Jang and Dolan, 2011). Whereas PpRIC overexpression (ca. 40x WT Pp*RIC* expression level) does not substantially affect directional cell expansion (Ntefidou et al., 2023) and therefore appears to directly block caulonema differentiation, PpROP1 overexpression

(ca. 30x increase in total Pp*ROP* gene expression as compared to WT; Fig. 6A)

presumably interferes with this process also indirectly by disrupting tip growth. Similarly,

the inhibition of caulonema differentiation observed at PpROP activity levels lower than

those displayed by *rop*^{2xKO} mutants appears more likely to be a consequence of severely

reduced directional cell expansion (Fig. 9) than to indicate a direct role of low-level

PpROP activity in the stimulation of caulonema differentiation.

PpROP activity contributes to the maintenance of apical initial cell identity

Protonemal filaments elongate based on tip growth and regular division of apical initial

cells, which undergo gradual caulonema differentiation. PpROP activity appears to play

an important role in the maintenance of apical initial cell identity not only by stimulating

tip growth and by inhibiting caulonema differentiation as discussed above, but also by

promoting the rate of cell division. Supporting this notion and consistent with previously

reported functions of mammalian RHO GTPases in stimulating cell proliferation

(Coleman et al., 2004), cell division rates were increasingly reduced in *rop*^{3xKO} and in

rop^{4xKO} mutants (Fig. 9). As rapid cell expansion was proposed to enhance the

proliferation of apical protonemal cells (Fantes and Nurse, 1977; Jones et al., 2017),

PpROP activity may promote the division of these cells indirectly by increasing the rate

of tip growth, rather than directly by accelerating cell cycle progression. Alternatively,

PpROP activity may stimulate both these processes.

PpROP activity induces the formation of gametophore-like structures and

promotes the growth of regular gametophores

Regular gametophore formation was entirely abolished by high-level PpROP

overexpression, as well as at levels of total PpROP activity lower than those displayed

by *rop*^{3xKO} mutants (Fig. 9). The same conditions also completely blocked caulonema

differentiation (Fig. 9), which was proposed to be essential for regular gametophore

formation (Cove and Knight, 1993; Schumaker and Dietrich, 1997; Brun et al., 2003;

Harrison et al., 2009). Strongly enhanced or reduced PpROP activity therefore appears

to indirectly prevent regular gametophore formation by disrupting caulonema

differentiation. However, in liquid culture medium estradiol-induced PpROP2 expression

at low levels insufficient for regular gametophore formation, or PpROP1^{Q64L} expression

at endogenous level, induced and supported the development of gametophore like-

structures composed of rudimentary phyllids without rhizoids, which were never formed
in the complete absence of PpROP activity (Fig. 9). Whereas regular gametophores
develop from caulonema cells undergoing a well-defined series of asymmetric and
symmetric cell divisions (Moody et al., 2018), gametophore like-structures emerged from
undifferentiated or chloronemal cells dividing in a comparably random fashion (Fig. 9;
Supplementary Fig. S8). Despite these differences, the observation that low-level
PpROP activity can induce the formation of aberrant gametophore-like structures
suggests that at higher levels enabling caulonema differentiation, this activity may also
play an important role in initiating regular gametophore development. Furthermore,
regular gametophore growth clearly depends on PpROP activity, since rop^{2xKO} and
rop^{3xKO} mutants, as well as complemented rop^{4xKO} mutants displaying similar levels of
total PpROP activity, formed gametophores with small leafy shoots and stunted rhizoids
(Fig. 9). These observations demonstrate that PpROP activity not only has essential
functions in promoting tip growth responsible for the elongation of protonemal filaments
and rhizoids, but also plays an apparently somewhat less important role in stimulating
other forms of directional cell expansion, which are required for the growth of leafy
shoots.

GDP/GTP cycling is essential for PpROP functions in fundamental developmental
processes

When expressed at endogenous level in rop^{4xKO} mutants, both WT PpROP2 and fast-
cycling $PpROP1^{F31L}$ restored the rop^{3xKO} phenotype. By contrast, at the same
expression level GTP-locked $PpROP1^{Q64L}$ was unable to complement defects in cell
polarization and parallel cell plate positioning displayed by rop^{4xKO} mutants, but induced
the formation of gametophore-like structures by these mutants in liquid medium (Fig. 9).
These observations demonstrate that GDP/GTP cycling is essential for PpROP
functions in fundamental developmental processes underlying filament formation, but is
not necessary for the induction of gametophore-like structures or directional cell
expansion responsible for the growth of these structures. Further investigations are
required to determine, whether GDP/GTP cycling is also essential for other PpROP
functions such as the promotion of tip growth, which requires cell polarization, or the
inhibition of caulonema differentiation, which depends on the formation of elongating

filaments. While RHO GTPase functions generally appear to depend on GDP/GTP
cycling, in animals atypical endogenous RHO GTPase have been identified, which like
PpROP1^{Q64L} regulate selected cellular or developmental processes in a constitutively
GTP-bound conformation (Hodge and Ridley, 2016; Aspenström, 2022).

Evolutionary origin and maintenance of the Pp*ROP* gene family

P. patens protonemata express at similar levels four Pp*ROP* genes encoding nearly
identical proteins, which as demonstrated by data presented here have essentially
redundant functions and together provide total PpROP activity that governs key
processes underlying protonemal development. A similar degree of sequence
conservation and functional integration is not generally observed in eukaryotic gene
families and raises questions concerning origin and maintenance of the Pp*ROP* gene
family during evolution.

At least one *ROP* gene is generally expressed in all plants (viridiplantae) with the
exception of some green algae of the chlorophyte group (Mulvey and Dolan, 2023b, a;
Ntefidou et al., 2023). Interestingly, *ROP* gene families typically have between four and
more than ten members in ferns, gymnosperm, and flowering plants, but are much
smaller with only one or two members in most plants displaying more ancient features,
which include lycophytes, the simplest vascular plants, along with mosses and all other
non-vascular plants (Mulvey and Dolan, 2023a). Similarly, *RIC* gene families also
substantially expanded and structurally differentiated with the emergence of flowering
plants (Ntefidou et al., 2023). These observations suggest a) that the evolution of
structurally increasingly complex vascular plants was accompanied by the diversification
of ROP functions, and b) that one or two ROP isoforms are generally sufficient to exert
comparably simple functions in plants with more ancient features. Consequently, total
PpROP activity regulating *P. patens* protonemal development, which is provided by
four co-expressed, nearly identical and functionally redundant PpROP family members,
appears to correspond to the activity of one or two ROP isoforms in other non-vascular
plants and in lycophytes.

The four members of the Pp*ROP* family are likely to have originated from a single
ancient ROP gene as a consequence of two rounds of whole genome duplications

(WGDs), which have occurred during the evolution of the *P. patens* genome (Rensing et
al., 2007; Lang et al., 2018; Leebens-Mack et al., 2019; Gao et al., 2022). Supporting
this notion, two consecutive WGDs and *ROP* gene families with four members have also
been identified in mosses of the genus *Sphagnum*, whereas more than one WGD
seems to have rarely occurred during the evolution of non-vascular plants with smaller
ROP gene families (Supplementary Table S2; Lang et al., 2018; Leebens-Mack et al.,
2019; Gao et al., 2022; Mulvey and Dolan, 2023b, a). The four *Sphagnum fallax* ROPs
and all ROPs expressed in a number of mosses with smaller *ROP* gene families share
at least 93 % identical amino acids with PpROP1 (Supplementary Table S2), suggesting
that ROP functions are highly conserved in all mosses, which is in line with the
hypothesis formulated at the end of the previous paragraph.

Remarkably, the four Pp*ROP* genes encoding nearly identical proteins, which apparently
derived from genome duplications, were maintained in *P. patens* evidently in the
absence of selective pressure driving their functional diversification. Although some
degree of functional redundancy commonly appears to be evolutionary stable,
particularly in gene families with important signaling functions (Vieten et al., 2005; Cevik
et al., 2023; Kumar and Ruiz, 2023), the selective advantage of this phenomenon is not
fully understood (Dean et al., 2008) and subject of ongoing further investigation (Nowak
et al., 1997; Raval et al., 2023). Genomic functional redundancy can provide resilience
against loss-of-function mutations (Gu et al., 2003b), as exemplified by the essentially
normal development of *P. patens rop*^{1xKO} mutants. Furthermore, it increases gene
dosage, which was proposed to enhance plant adaptability to changing environmental
conditions (Kondrashov et al., 2002).

Outlook
Evolutionary conserved essential functions of ROP GTPases in cell polarization,
directional cell expansion and cell plate positioning have previously been established
based on the investigation of defects caused by loss-of-function or gain-of-function
mutations in the moss *P. patens* (Burkart et al., 2015; Cheng et al., 2020; Yi and
Goshima, 2020; Bao et al., 2022), in the closely related non-vascular liverwort *M.*
polymorpha (Mulvey and Dolan, 2023b), as well as in the flowering plants *A. thaliana*
(Roszak et al., 2021) and *Z. mays* (Humphries et al., 2011). Data shown here

demonstrate that during *P. patens* protonemal development PpROP activity has
additional important functions in inhibiting caulonema differentiation, in controlling
gametophore development, and in the direct or indirect regulation of cell proliferation.
Whether ROP GTPases have similar or related functions also in other plants represents
an important area of future research.

The discoveries summarized in the previous paragraph have provided an increasingly
detailed understanding of ROP functions in the control of different cellular and
developmental processes in plants. However, the signaling network stimulated by ROP
activity to regulate these processes has not been well characterized. Compared to ROP
and RHO GTPase functions, this network appears to be much less evolutionary
conserved. In line with this notion, data presented here demonstrate that AtROP7 and
HsRHOA can only complement the rop^{4xKO} phenotype to a very limited extent. Although
these heterologous RHO GTPases are closely related to PpROPs (85.2-85.7 %
[AtROP7] and 49.7 % [HsRHOA] amino acid identity) even upon massive
overexpression they are only able to restore developmental processes in rop^{4xKO}
mutants, which require minimal PpROP activity. Consistent with this finding, homologs of
key animal RHO downstream effectors such as PAKs or ROCKs seem to be generally
missing in plants. Instead, plant-specific ROP effectors including RICs, ICR/RIPs and
RISAPs were shown to control directional cell expansion in flowering plants (Lavy et al.,
2007; Yalovsky et al., 2008; Stephan et al., 2014). Interestingly, the *P. patens* genome
encodes no ICR/RIP or RISAP homologs and only a single member of the RIC family
(Eklund et al., 2010; Ntefidou et al., 2023). Furthermore, the single PpRIC protein is
structurally very different from all its 11 *A. thaliana* homologs, and unlike several of these
proteins does not regulate directional cell expansion, but inhibits caulonema
differentiation (Ntefidou et al., 2023). Consequently, nothing is currently known about the
effectors regulating tip growth downstream of PpROP activity in *P. patens*, and PpRIC is
the only PpROP effector with a demonstrated function in the control of other cellular or
developmental processes in this moss (Fig. 9). To further characterize the signaling
network activated by PpROPs, it will be essential to identify and functionally characterize
novel interaction partners and potential effectors of these proteins. The same approach

will also provide important insights into the adaptation of ROP downstream signaling

during plant evolution.

Materials and Methods

Plant materials and culture conditions

P. patens ecotype Gransden wild-type (WT) strain (2012) (Ashton and Cove, 1977) and

transgenic lines (Supplementary Table S3) were grown axenically at 25°C under

continuous white light illumination with fluorescent tubes (Philips Master TL-D Super 80

58 W, cool white) at an intensity of 50 µmol m^{-2} s^{-1}. Unless otherwise stated, moss

protonemata were cultivated and analyzed in 9-mm Petri dishes on BCDA medium (1

mM $MgSO_4$, 1.84 mM KH_2PO_4, 10 mM KNO_3, 5 mM ammonium-tartrate, 1 mM $CaCl_2$,

45 µM $FeSO_4$, 9.9 µM H_3BO_3, 2 µM $MnCl_2$, 116 nM $AlK[SO_4]_2$, 424 nM $CoCl_2$, 220 nM

$CuSO_4$, 235 nM KBr, 168 nM KI, 660 nM LiCl, 124 nM $SnCl_2$, and 191 nM $ZnSO_4$)

(Ashton and Cove, 1977) solidified with 0.7% (w/v) agar and supplemented with 100

µg/mL vancomycin (Duchefa Biochemie) unless other antibiotics were used for

transgene selection. Solidified medium was covered with cellophane discs (AA

Packaging) (Grimsley et al., 1977) except for long-term cultivation of moss cultures

consisting of small clumps of round cells. Protonemata were sub-cultured every 7 days

and regenerated every 6 weeks from gametophores by homogenization (OMNI

International) in Milli-Q H_2O (Merck). Gametophore-forming colonies were cultivated and

analyzed on BCD medium (BCDA without ammonium-tartrate, supplemented with 100

µg/ml vancomycin) solidified with 0.7% (w/v) agar or in liquid BCD medium. Titratable

expression of RHO GTPases was induced by adding β-estradiol (Sigma), dissolved in

DMSO at concentrations from $2x10^{-5}$ µM to 1 µM to BCDA or BCD medium after

autoclaving. The culture conditions used in each experiment are listed in Supplementary

Table S4.

Phenotypic analysis of protonemata regenerated from protoplasts

Protonemata regenerated from protoplasts were prepared as described previously (Le

Bail et al., 2019) based on Cove et al. (Cove et al., 2009). In brief, 4 to 7-day-old

protonemata were digested with 0.5% (w/v) driselase (Sigma) in 8.5% mannitol and

regenerated on PRMB medium (supplemented with 100 µg/ml vancomycin) solidified

with 0.7 % agar in Petri dishes covered with cellophane for 2 days, followed by transfer

of the cellophane disc to BCDA medium (supplemented with 100 µg/ml vancomycin).

BCDA medium was treated with ≤ 1 µM β-estradiol for Pp*ROP1* overexpression

(WT/*ind*^pro:*ROP1*) or complementation of *rop*^4xKO with an inducible *ROP* or *RHO*

expression construct (*rop*^4xKO/*ind*^pro:ROPX or *rop*^4xKO/*ind*^pro:*RHO*). Nuclei were counted 4

days after protoplast preparation. Cell length, caulonema differentiation and protonemal

area were measured at 5 days. To analyze the gametophore stage, individual

protonemal colonies, having regenerated for 5 days on BCDA were transferred to BCD

medium without cellophane and supplemented with 100 µg/ml vancomycin, and the

colony area was measured 4 or 5 weeks after protoplast preparation. The culture

conditions used in each experiment are listed in Supplementary Table S4. Experiments

were repeated three times. The cell length and the area of 5-day-old and 5-week-old

colonies in Figures 1, 2, 4, and Supplementary Figure S5 were analyzed simultaneously.

Bright field microscopy of cell length, caulonema differentiation, and colony size

To determine the cell length and caulonema differentiation, filaments with three or more

cells of 5-day-old protonemata regenerated from protoplasts were imaged using a wide-

field microscope (DMI4000B, Leica). The length of subapical cells was analyzed using

Fiji software (ImageJ 1.54f; Schindelin et al., 2012), and caulonema differentiation was

assessed by optical inspection of apical cells. The colony size was determined based on

chlorophyll autofluorescence (excitation, 470 nm; emission, 500 nm long-pass) images

obtained with a fluorescence stereo microscope (M205 FA, Leica) and analyzed using a

Fiji macro (Vidali et al., 2007). 4 to 5-week-old colonies were imaged using the stereo

microscope (M205 FA, Leica), and the area was determined by applying thresholding to

convert reflected light 8-bit images to 1-bit images and analyzed using Fiji software

(ImageJ 1.54f; Schindelin et al., 2012).

Generation of knockout constructs

Molecular cloning was performed with standard methods (Green and Sambrook, 2012)

using Phusion DNA polymerase (ThermoFisher Scientific), primers listed in

Supplementary Data Set S1 (Eurofins Genomics), restriction enzymes and T4 Ligase

(New England Biolabs). The resulting constructs (Supplementary Data Set S2) were

verified by Sanger sequencing (Eurofins Genomics). To knock out Pp*ROP*s (*rop1, rop2,*

rop3, and *rop4*) based on homologous recombination, genomic fragments of 0.7 to 1.0

kb upstream of the start codon (5′ target) and downstream of the stop codon (3′ target)

were cloned flanking a resistance marker. The backbone of pMT123 (Thelander et al.,

2004) was used for cloning into the XhoI/HindIII and SpeI/NotI sites, resulting in pSLU33

for knockout of Pp*ROP1* (Supplementary Fig. S2A and B); the p35S-Zeo backbone

(Hiwatashi et al., 2008) was used for cloning pSLU34 for knockout of Pp*ROP2*

(Supplementary Fig. S3A and B), the p35S-loxP-BSD backbone (Li et al., 2017) was

used for cloning pSLU35 for knockout of Pp*ROP3* (Supplementary Fig. S3C and D), and

the pMT164 backbone (Thelander et al., 2007) was used for cloning pSLU36 for

knockout of Pp*ROP4* (Supplementary Fig. S4). Multiple Pp*rop* knockouts (rop^{2xKO},

rop^{3xKO}, rop^{4xKO}) were generated through sequential knockout of single PpROPs in the

order listed in Supplementary Fig. S2C. For each knockout, at least two lines were

obtained except for *rop2, rop4,* and *rop4/1,* which had only one line each

(Supplementary Table S3). All lines for each genotype showed the same phenotype, and

the results of only one transgenic line are presented.

Generation of expression constructs

To complement rop^{4xKO} with Pp*ROP1*^{Q64L} or Pp*ROP1*^{F31L} expressed by the endogenous

Pp*ROP1* promoter, the Pp*ROP1* coding and 3'UTR sequence were amplified from cDNA

and cloned into pENTR™/D-Topo (Thermo Fisher Scientific) yielding pFAU253 and

mutated using PCR-based site-directed mutagenesis followed by DpnI digestion

generating pFAU256 (Pp*ROP1*^{Q64L}) or pFAU417 (Pp*ROP1*^{F31L}). The Pp*ROP1*^{Q64L} or

Pp*ROP1*^{F31L} fragments were transferred into the SalI/HindIII sites of pMT123 (Thelander

et al., 2004) and flanked by 0.7 to 1.0 kb regions upstream of the start codon (5′ target)

or downstream of the stop codon (3′ target) of Pp*ROP1* enabling the replacement of the

Pp*ROP1* locus in *rop3/2/4* through homologous recombination yielding pFAU306

(rop^{4xKO}/*ROP1*^{pro}:*ROP1*^{Q64L}) or pFAU421 (rop^{4xKO}/*ROP1*^{pro}:*ROP1*^{F31L}) (Supplementary

Fig. S2A and B; Supplementary Data Set S2).

To complement rop^{4xKO} with a single Pp*ROP* under the control of the Pp*ROP1* promoter,

the full-length coding sequences of Pp*ROP2* or Pp*ROP3,* including the respective 3′

UTR, were amplified from cDNA and cloned into pENTR™/D-Topo, generating pFAU269

(Pp*ROP2*) and pFAU270 (Pp*ROP3*). Each Pp*ROP* fragment was then transferred from

pFAU253 or pFAU269 or pFAU270 into the SalI/HindIII sites of pFAU306 which enabled

the replacement of the Pp*ROP1* locus in *rop3/2/4* through homologous recombination,

yielding pFAU508 (*rop*[4xKO]/*ROP1*[pro]:*ROP1*), pFAU509 (*rop*[4xKO]/*ROP1*[pro]:*ROP2*), and

pFAU517 (*rop*[4xKO]/*ROP1*[pro]:*ROP3*) respectively (Supplementary Fig. S2A and B).

To complement *rop*[4xKO] with a titratable expression of a *ROP* or *RHO GTPase*, the lexA

operator and the XVE chimeric sequence were amplified by PCR from pGX8 (Kubo et

al., 2013) and cloned into the NotI/EcoRV sites of pSLU35, yielding pFAU425, and into

the SpeI/PmeI/PmlI sites of pSLU36, yielding pFAU391. The coding and 3' UTR

sequence of Pp*ROP2* were transferred from pFAU269 to pFAU425 by Gateway LR

reaction (Thermo Fisher Scientific) (Katzen, 2007) generating pFAU431

(*rop*[4xKO]/*ind*[pro]:*ROP2*) (Supplementary Fig. S3C and D) that was used for replacement of

the Pp*ROP3* locus based on homologous recombination in *rop2/4/1*. The full-length

At*ROP7* and Hs*RHOA* coding sequences were amplified from Arabidopsis or *Homo*

sapiens cDNA, respectively, and cloned into pENTR/D-Topo (Thermo Fisher Scientific),

yielding pFAU290 (At*ROP7*) and pFAU294 (Hs*RHOA*), followed by Gateway LR reaction

(Thermo Fisher Scientific) (Katzen, 2007) with pFAU391 generating pFAU398

(*rop*[4xKO]/*ind*[pro]:At*ROP7*) or pFAU402 (*rop*[4xKO]/*ind*[pro]:Hs*RHOA*) for gene replacement

based on homologous recombination of the Pp*ROP4* locus in *rop3/1/2* (Supplementary

Fig. S4A and B).

To overexpress Pp*ROP1* (WT/*ind*[pro]:*ROP1*) from the β-estradiol-inducible promoter, the

coding sequence of Pp*ROP1*, including the 3' UTR, was transferred from pFAU253 by

Gateway LR reaction (Thermo Fisher Scientific) (Katzen, 2007) into pGX8 (Kubo et al.,

2013) generating pFAU461 (Supplementary Fig. S2D) used for transgene integration

based on homologous recombination into the neutral genomic region of PIG1b (Okano

et al., 2009).

To express Pp*PINA-eGFP* under the endogenous Pp*PINA* promoter, the pFAUobt63

vector was obtained from Mattias Thelander (Viaene et al., 2014) for integration of

PINA[pro]:*PINA-eGFP* into the neutral genomic region Pp108 (Schaefer and Zrÿd, 1997) of

P. patens WT and *rop1/2* double knockout.

***P. patens* transformation**

Gene targeting into the *P. patens* genome by homologous recombination was previously
described for polyethylene glycol PEG-mediated transformation (Le Bail et al., 2019)
based on the method by Schaefer and Zrÿd (Schaefer and Zrÿd, 1997). In brief, 1.6 x
10^6 protoplasts were transformed with 15 µg of linearized vector and allowed to
regenerate for 5 days on PRMB medium, followed by two rounds of selection alternating
between BCDA medium containing the appropriate antibiotic (30 µg/mL hygromycin B
[Carl Roth], 20 µg/mL G418 [Merck], 50 µg/mL zeocin [Thermo Fisher Scientific], or 75
µg/mL blasticidin [InvivoGen]) according to the transformed resistance cassette and
BCDA medium (supplemented with 100 µg/ml vancomycin). Transgenic lines were
genotyped through PCR using primers indicated in Supplementary Data Set S1 and
Supplementary Fig. S2-S4.

Gene expression analysis by RT-qPCR
Total RNA was extracted from 1-week-old protonemata cultivated through
homogenization or from 7 to 10-day-old protonemata regenerated from protoplasts.
Samples were frozen in liquid nitrogen and lysed with glass beads using TissueLyser II
(Qiagen). Total RNA was isolated using a Nucleospin RNA Plus^{TM} Kit (Macherey-Nagel),
and RNA integrity was assessed using agarose gel electrophoresis. First-strand cDNA
was synthesized from 1 µg of total RNA using iScript^{TM} Reverse Transcription Supermix
for RT-qPCR (Bio-Rad) and diluted 1:20 in Milli-Q H_2O. RT-qPCR was conducted using
SscAdvanced^{TM} Universal SYBR Green Supermix (Bio-Rad) and a CFX96TM thermal
cycler (Bio-Rad). Total RNA was prepared from three moss samples (biological
replicates), and each cDNA sample was measured in two RT-qPCR reactions (technical
replicates). Absolute transcript levels were quantified with standard curves prepared
from a 10-fold dilution series of genomic DNA from 1-week old protonemata using the
PhytoPure DNA Extraction kit (Cytiva). Pp*UBIQUITIN-E2* served as a reference gene
(Le Bail et al., 2013). Primers used for RT- qPCR are listed in Supplementary Data Set
S1. Experiments were repeated two times.

Quantification of free IAA
7-day-old protonemata tissues (around 15-30 mg fresh weight per sample) were
extracted after addition of 500 pg ^{13}C6-IAA internal standard per sample, purified and

analyzed for free IAA concentration using combined gas chromatography-tandem mass

spectrometry as described (Andersen et al., 2008).

Confocal microscopy and staining of nuclei and cell walls

A Leica TCS SP8 DIVE-FALCON inverted confocal laser scanning microscope with an

HC PL APO CS2 20X/0.75 NA water immersion lens was used for confocal imaging,

operated by Application Suite X software. GFP fluorescence was imaged through

excitation using an argon laser at 488 nm and emission at 500-550 nm. The cell number

was determined by counting nuclei in 4-day-old cells, or protonemata regenerated from

protoplasts, stained according to the method of Vidali et al. (Vidali et al., 2010). In brief,

moss samples were fixed for 30 min in a staining solution of 100 mM PIPES, pH 6.8,

0.1% (v/v) Nonidet P-40, 2% (w/v) paraformaldehyde, and 0.1 µg/mL 4′,6-diamidino-

2phenylindole (DAPI). The fluorescence was detected through excitation using an argon

laser at 360 nm and emission at 460 nm. Cell walls of gametophores were stained with

10 µg/mL propidium iodide solution (Merck), and the fluorescence was excited using a

DPSS 561 laser (excitation, 536 nm; emission, 617 nm) and recorded using Z-stacks of

optical sections at 2-µm spacing. Fiji software (Schindelin et al., 2012) was used to

generate maximum Z-stack projections.

Alignment analysis

A protein alignment of full-length PpROPs, AtROP7, and HsRHOA sequences and the

protein identity matrix were obtained using Uniprot (The UniProt Consortium, 2024).

Statistical analysis

GraphPad Prism (Version 10.3.1, GraphPad software) was used for data analysis.

Unpaired Student's *t*-tests were used to determine significant differences between two

genotypes and one-way analysis of variance (ANOVA, Welch's ANOVA) or two-way

ANOVA followed by pairwise multiple comparison tests (Tukey's/Dunnett's) between

three or more genotypes as indicated in the figure legends. Significance to WT and

comparisons relevant to data interpretation are indicated in graphs, and all statistical

results are given in Supplementary Data Set S3.

Accession numbers

Sequence data were analyzed by BLAST (Altschul et al., 1990) and are available in the data libraries of Phytozome *P. patens* V6.1 (Joint Genome Institute) (Goodstein et al., 2012; Bi et al., 2024), TAIR (Arabidopsis Information Resource) (Berardini et al., 2015), ONEKP CNGBdb (Leebens-Mack et al., 2019), and GenBank (Clark et al., 2016) under the following accession numbers: Aa*ROP* (onekp|ZTHV_scaffold_2084304 Atrichum_angustatum, CNGBdb); Aat*ROP1* (onekp|QMWB_scaffold_2004844 Anomodon_attenuatus, CNGBdb); Aat*ROP2* (onekp|QMWB_scaffold_2004846 Anomodon_attenuatus, CNGBdb); Aat*ROP3* (onekp|QMWB_scaffold_2004845 Anomodon_attenuatus, CNGBdb); Ar*ROP* (gnl|onekp|WOGB_scaffold_2094631 Andreaea_rupestris, CNGBdb); At*ROP7* (At5g45970, TAIR); Ba*ROP* (gnl|onekp|JMXW_scaffold_2006533 Bryum_argenteum, CNGBdb); Bap*ROP* (gnl|onekp|HRWG_scaffold_2070290 Buxbaumia_aphylla, CNGBdb); Cc*ROP* (gnl|onekp|TAVP_scaffold_2005151 Calliergon_cordifolium, CNGBdb); Cd*ROP* (gnl|onekp|MIRS_scaffold_2008952 Climacium_dendroides, CNGBdb); Cp*ROP1* (gnl|onekp|FFPD_scaffold_2008289 Ceratodon_purpureus, CNGBdb); Cp*ROP2* (gnl|onekp|FFPD_scaffold_2009252 Ceratodon_purpureus, CNGBdb); Df*ROP* (gnl|onekp|AWOI_scaffold_2072508 Diphyscium_foliosum, CNGBdb); Fa*ROP* (gnl|onekp|DHWX_scaffold_2074728 Fontinalis_antipyretica, CNGBdb) Hs*RHOA* (NM_001664.4, GenBank); La*ROP* (gnl|onekp|VMXJ_scaffold_2010101 Leucobryum_albidum, CNGBdb); Ol*ROP1* (gnl|onekp|CMEQ_scaffold_2011516 Orthotrichum_lyellii, CNGBdb); Ol*ROP2* (gnl|onekp|CMEQ_scaffold_2013049 Orthotrichum_lyellii, CNGBdb); Pf*ROP* (gnl|onekp|ORKS_scaffold_2003761 Philonotis_fontana, CNGBdb); Pc*ROP* (onekp|SZYG_scaffold_2042265 Polytrichum_commune, CNGBdb); Pp*AUX1* (Pp6c16_1210, Phytozome); Pp*PINA* (Pp6c23_4450, Phytozome); Pp*ROP1* (Pp6c14_2140, Phytozome); Pp*ROP2* (Pp6c2_11900, Phytozome); Pp*ROP3* (Pp6c1_11060, Phytozome); Pp*ROP4* (Pp6c10_2660, Phytozome); Pp*RSL1 (*Pp6c1_20350, Phytozome); Pp*SHI1* (Pp6c21_9000, Phytozome); and Pp*UBIQUITIN-E2* (Pp6c12_2450, Phytozome); Rs*ROP1* (gnl|onekp|JADL_scaffold_2005567 Rhynchostegium_serrulatum, CNGBdb); Rs*ROP2 (gnl|onekp|JADL_scaffold_2005565 Rhynchostegium_serrulatum*, CNGBdb); Sf*ROP1* (Sphfalx06G097600, Phytozome); Sf*ROP2* (Sphfalx13G088000, Phytozome); Sf*ROP3* (Sphfalx14G076800, Phytozome); Sf*ROP4* (Sphfalx18G051700, Phytozome); Tl*ROP* (gnl|onekp|SKQD_scaffold_2079078 Takakia_lepidozioides, CNGBdb).

Supplementary Data

Supplementary Fig. S1. PpROP gene expression in protonemata.

Supplementary Fig. S2. Editing of the genomic Pp*ROP1* locus through homologous recombination and summary of transgenic lines.

Supplementary Fig. S3. Editing of the genomic Pp*ROP2* and Pp*ROP3* loci through homologous recombination.

Supplementary Fig. S4. Editing of the genomic Pp*ROP4* locus through homologous

recombination.

Supplementary Fig. S5. Knockout of a single Pp*rop* (*rop*[1xKO]) does not affect

protonemata.

Supplementary Fig. S6. PpROPs do not influence the expression of auxin-regulated

gene expression or the auxin content.

Supplementary Fig. S7. β-estradiol does not influence Pp*ROP* expression or

protonemal development.

Supplementary Fig. S8. Gametophore-like structures of *rop*[4xKO]/*ROP1*[pro]:*ROP1*^{Q64L}.

Supplementary Table S1. Rate of polarized cell expansion of *rop*4xKO/*ind*[pro]:*ROP2* at

low induction level.

Supplementary Table S2. ROP protein families in mosses: number of members and

amino acid sequence conservation.

Supplementary Table S3. *P. patens* lines used in this study.

Supplementary Table S4. Culture media used in this study.

Supplementary Data Set 1. Oligonucleotides used in this study.

Supplementary Data Set 2. Vectors used in this study.

Supplementary Data Set 3. Statistical analyses.

Author contributions

Designed the research: A.L.B, M.N., and B.K.; Performed research: A.L.B., M.N., J.N.,

T.I.L., D.K., K.L., H.V., S.S.; Data analysis: A.L.B., B.K., M.N., K.L.; Writing – original

draft: M.N.; Writing – review & editing: B.K., M.N.; Visualization: M.N.; Supervision: B.K.

and M.N.; Project administration: B.K.; Funding acquisition: K.L. and B.K. All authors are

in agreement with this MS and approved its content.

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References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. J. Mol. Biol. **215,** 403-410. 10.1016/S0022-2836(05)80360-2

Andersen, S.U., Buechel, S., Zhao, Z., Ljung, K., Novák, O., Busch, W., Schuster, C., and Lohmann, J.U. (2008). Requirement of B2-type cyclin-dependent kinases for meristem integrity in *Arabidopsis thaliana*. Plant Cell **20,** 88-100. https://doi.org/10.1105/tpc.107.054676

Ashton, N.W., and Cove, D.J. (1977). The isolation and preliminary characterisation of auxotrophic and analogue resistant mutants of the moss, *Physcomitrella patens*. Mol. Gen. Genetics **154,** 87-95. https://doi.org/10.1007/BF00265581

Aspenström, P. (2019). The Intrinsic GDP/GTP Exchange Activities of Cdc42 and Rac1 Are Critical Determinants for Their Specific Effects on Mobilization of the Actin Filament System. Cells **8,** 759. 10.3390/cells8070759

Aspenström, P. (2022). The Role of Fast-Cycling Atypical RHO GTPases in Cancer. Cancers **14,** 1961. https://doi.org/10.3390/cancers14081961

Bao, L., Ren, J., Nguyen, M., Slusarczyk, A.S., Thole, J.M., Martinez, S.P., Huang, J., Fujita, T., and Running, M.P. (2022). The cellular function of ROP GTPase prenylation is important for multicellularity in the moss *Physcomitrium patens*. Development **149**. https://doi.org/10.1242/dev.200279

Berardini, T.Z., Reiser, L., Li, D., Mezheritsky, Y., Muller, R., Strait, E., and Huala, E. (2015). The arabidopsis information resource: Making and mining the "gold standard" annotated reference plant genome. genesis **53,** 474-485. https://doi.org/10.1002/dvg.22877

Berken, A., Thomas, C., and Wittinghofer, A. (2005). A new family of RhoGEFs activates the Rop molecular switch in plants. Nature **436,** 1176-1180. https://doi.org/10.1038/nature03883

Bi, G., Zhao, S., Yao, J., Wang, H., Zhao, M., Sun, Y., Hou, X., Haas, F.B., Varshney, D., Prigge, M., Rensing, S.A., Jiao, Y., Ma, Y., Yan, J., and Dai, J. (2024). Near telomere-to-telomere genome of the model plant Physcomitrium patens. Nat. Plants **10,** 327-343. 10.1038/s41477-023-01614-7

Bishop, A.L., and Hall, A. (2000). Rho GTPases and their effector proteins. Biochem. J. **348,** 241-255. 10.1042/bj3480241

Boureux, A., Vignal, E., Faure, S., and Fort, P. (2007). Evolution of the Rho family of ras-like GTPases in eukaryotes. Mol. Biol. Evol. **24,** 203-216. 10.1093/molbev/msl145

Brun, F., Gonneau, M., Laloue, M., and Nogué, F. (2003). Identification of Physcomitrella patens genes specific of bud and gametophore formation. Plant Sci. **165,** 1267-1274. https://doi.org/10.1016/S0168-9452(03)00335-2

Burkart, G.M., Baskin, T.I., and Bezanilla, M. (2015). A family of ROP proteins that suppress actin dynamics and are essential for polarized growth and cell adhesion. J. Cell Sci. **128,** 2553-2564. https://doi.org/10.1242/jcs.172445

Cevik, S., Peng, X., Beyer, T., Pir, M.S., Yenisert, F., Woerz, F., Hoffmann, F., Altunkaynak, B., Pir, B.,
Boldt, K., Karaman, A., Cakiroglu, M., Oner, S.S., Cao, Y., Ueffing, M., and Kaplan, O.I. (2023). WDR31
displays functional redundancy with GTPase-activating proteins (GAPs) ELMOD and RP2 in regulating IFT
complex and recruiting the BBSome to cilium. Life Sci. Alliance **6,** e202201844.
https://doi.org/10.26508/lsa.202201844
Chen, C.Y.H., Cheung, A.Y., and Wu, H.-M. (2003). Actin-Depolymerizing Factor Mediates Rac/Rop
GTPase–Regulated Pollen Tube Growth. Plant Cell **15,** 237-249. https://doi.org/10.1105/tpc.007153
Cheng, X., Mwaura, B.W., Chang Stauffer, S.R., and Bezanilla, M. (2020). A fully functional ROP
fluorescent fusion protein reveals roles for this GTPase in subcellular and tissue-level patterning. Plant
Cell **32,** 3436-3451. https://doi.org/10.1105/tpc.20.00440
Clark, K., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J., and Sayers, E.W. (2016). GenBank. Nucleic Acids
Res. **44,** D67-D72. https://doi.org/10.1093/nar/gkv1276
Coleman, M.L., Marshall, C.J., and Olson, M.F. (2004). RAS and RHO GTPases in G1-phase cell-cycle
regulation. Nat. Rev. Mol. Cell Biol. **5,** 355-366. 10.1038/nrm1365
Collonnier, C., Epert, A., Mara, K., Maclot, F., Guyon-Debast, A., Charlot, F., White, C., Schaefer, D.G.,
and Nogué, F. (2017). CRISPR-Cas9-mediated efficient directed mutagenesis and RAD51-dependent and
RAD51-independent gene targeting in the moss *Physcomitrella patens*. Plant Biotechnol. J. **15,** 122-131.
10.1111/pbi.12596
Cove, D. (2005). The Moss *Physcomitrella patens*. Annu. Rev. Genet. **39,** 339 - 358.
10.1146/annurev.genet.39.073003.110214
Cove, D., and Knight, C. (1993). The moss *Physcomitrella patens,* a model system with potential for the
study of plant reproduction. Plant Cell **5,** 1483-1488. https://doi.org/10.1105/tpc.5.10.1483
Cove, D.J., Perroud, P.-F., Charron, A.J., McDaniel, S.F., Khandelwal, A., and Quatrano, R.S. (2009).
Isolation and Regeneration of Protoplasts of the Moss *Physcomitrella patens*. Cold Spring Harbor
Protocols **2009**. https://doi.org/doi:10.1101/pdb.prot5140
Dean, E.J., Davis, J.C., Davis, R.W., and Petrov, D.A. (2008). Pervasive and Persistent Redundancy among
Duplicated Genes in Yeast. PLOS Genet. **4,** e1000113. https://doi.org/10.1371/journal.pgen.1000113
Denninger, P., Reichelt, A., Schmidt, V.A.F., Mehlhorn, D.G., Asseck, L.Y., Stanley, C.E., Keinath, N.F.,
Evers, J.-F., Grefen, C., and Grossmann, G. (2019). Distinct RopGEFs Successively Drive Polarization and
Outgrowth of Root Hairs. Curr. Biol. **29,** 1854-1865. https://doi.org/10.1016/j.cub.2019.04.059
Eklund, D.M., Svensson, E.M., and Kost, B. (2010). *Physcomitrella patens:* a model to investigate the role
of RAC/ROP GTPase signalling in tip growth. J. Exp. Bot. **61,** 1917-1937.
https://doi.org/10.1093/jxb/erq080
Etienne-Manneville, S., and Hall, A. (2002). Rho GTPases in cell biology. Nature **420,** 629-635.
https://doi.org/10.1038/nature01148
Fantes, P., and Nurse, P. (1977). Control of cell size at division in fission yeast by a growth-modulated size
control over nuclear division. Exp. Cell Res. **107,** 377-386. https://doi.org/10.1016/0014-
4827(77)90359-7
Feiguelman, G., Fu, Y., and Yalovsky, S. (2018). ROP GTPases Structure-Function and Signaling Pathways.
Plant Physiol. **176,** 57-79. https://doi.org/10.1104/pp.17.01415
Fu, Y., Gu, Y., Zheng, Z., Wasteneys, G., and Yang, Z. (2005). Arabidopsis interdigitating cell growth
requires two antagonistic pathways with opposing action on cell morphogenesis. Cell **120,** 687-700.
10.1016/j.cell.2004.12.026
Gao, B., Chen, M.-X., Li, X.-S., Liang, Y.-Q., Zhang, D.-Y., Wood, A.J., Oliver, M.J., and Zhang, J.-H. (2022).
Ancestral gene duplications in mosses characterized by integrated phylogenomic analyses. J. Syst. Evol.
60, 144-159. https://doi.org/10.1111/jse.12683
Goodstein, D.M., Shu, S., Howson, R., Neupane, R., Hayes, R.D., Fazo, J., Mitros, T., Dirks, W., Hellsten,
U., Putnam, N., and Rokhsar, D.S. (2012). Phytozome: a comparative platform for green plant
genomics. Nucleic Acids Res. **40,** D1178-D1186. https://doi.org/10.1093/nar/gkr944

Green, M.R., and Sambrook, J. (2012). Molecular cloning: A laboratory manual. (Cold Spring Harbor, New
York: Cold Spring Harbor Laboratory Press).

Grimsley, N.H., Ashton, N.W., and Cove, D.J. (1977). The production of somatic hybrids by protoplast
fusion in the moss, *Physcomitrella patens*. Mol. Gen. Genet. **154,** 97-100.
https://doi.org/10.1007/bf00265582

Gu, Y., Vernoud, V., Fu, Y., and Yang, Z. (2003a). ROP GTPase regulation of pollen tube growth through
the dynamics of tip-localized F-actin. J. Exp. Bot. **54,** 93-101. 10.1093/jxb/erg035

Gu, Z., Steinmetz, L.M., Gu, X., Scharfe, C., Davis, R.W., and Li, W.-H. (2003b). Role of duplicate genes in
genetic robustness against null mutations. Nature **421,** 63-66. https://doi.org/10.1038/nature01198

Hall, A., and Lalli, G. (2010). Rho and Ras GTPases in axon growth, guidance, and branching. Cold Spring
Harb Perspect Biol **2,** a001818. https://doi.org/10.1101/cshperspect.a001818

Harrison, C.J., Roeder, A.H.K., Meyerowitz, E.M., and Langdale, J.A. (2009). Local Cues and Asymmetric
Cell Divisions Underpin Body Plan Transitions in the Moss *Physcomitrella patens*. Curr. Biol. **19,** 461-471.
https://doi.org/10.1016/j.cub.2009.02.050

Heasman, S.J., and Ridley, A.J. (2008). Mammalian Rho GTPases: new insights into their functions from
in vivo studies. Nat. Rev. Mol. Cell Biol. **9,** 690-701. 10.1038/nrm2476

Hiwatashi, Y., Obara, M., Sato, Y., Fujita, T., Murata, T., and Hasebe, M. (2008). Kinesins are
indispensable for interdigitation of phragmoplast microtubules in the moss *Physcomitrella patens*. Plant
Cell **20,** 3094-3106. https://doi.org/10.1105/tpc.108.061705

Hodge, R.G., and Ridley, A.J. (2016). Regulating Rho GTPases and their regulators. Nat. Rev. Mol. Cell
Biol. **17,** 496-510. https://doi.org/10.1038/nrm.2016.67

Humphries, J.A., Vejlupkova, Z., Luo, A., Meeley, R.B., Sylvester, A.W., Fowler, J.E., and Smith, L.G.
(2011). ROP GTPases Act with the Receptor-Like Protein PAN1 to Polarize Asymmetric Cell Division in
Maize. Plant Cell **23,** 2273-2284. https//doi.org.10.1105/tpc.111.085597

Ito, K., Ren, J., and Fujita, T. (2014). Conserved function of Rho-related Rop/RAC GTPase signaling in
regulation of cell polarity in *Physcomitrella patens*. Gene **544,** 241-247.
https://doi.org/10.1016/j.gene.2014.04.057

Jaeger, R., and Moody, L.A. (2021). A fundamental developmental transition in *Physcomitrium patens* is
regulated by evolutionarily conserved mechanisms. Evol. Devel. **23,** 123-136.
https://doi.org/10.1111/ede.12376

Jang, G., and Dolan, L. (2011). Auxin promotes the transition from chloronema to caulonema in moss
protonema by positively regulating PpRSL1and PpRSL2 in *Physcomitrella patens*. New Phytol. **192,** 319-
327. https://doi.org/10.1111/j.1469-8137.2011.03805.x

Jones, A.R., Forero-Vargas, M., Withers, S.P., Smith, R.S., Traas, J., Dewitte, W., and Murray, J.A.H.
(2017). Cell-size dependent progression of the cell cycle creates homeostasis and flexibility of plant cell
size. Nat. Commun. **8,** 15060. https://doi.org/10.1038/ncomms15060

Katzen, F. (2007). Gateway® recombinational cloning: a biological operating system. Expert Opin. Drug
Discov. **2,** 571-589. https://doi.org/10.1517/17460441.2.4.571

Kawano, Y., Kaneko-Kawano, T., and Shimamoto, K. (2014). Rho family GTPase-dependent immunity in
plants and animals. Front. Plant Sci. **5**. https://doi.org/10.3389/fpls.2014.00522

Klahre, U., and Kost, B. (2006). Tobacco RhoGTPase ACTIVATING PROTEIN1 Spatially Restricts Signaling of
RAC/Rop to the Apex of Pollen Tubes. Plant Cell **18,** 3033-3046.
https://doi.org/10.1105/tpc.106.045336

Kofuji, R., and Hasebe, M. (2014). Eight types of stem cells in the life cycle of the moss Physcomitrella
patens. Curr. Opin. Plant Biol. **17,** 13-21. 10.1016/j.pbi.2013.10.007

Kondrashov, F.A., Rogozin, I.B., Wolf, Y.I., and Koonin, E.V. (2002). Selection in the evolution of gene
duplications. Genome Biol. **3**. https://doi.org/10.1186/gb-2002-3-2-research0008

Kost, B. (2008). Spatial control of Rho (Rac-Rop) signaling in tip-growing plant cells. Trends Cell Biol. **18,**
119-127. 10.1016/j.tcb.2008.01.003

Kost, B. (2010). Regulatory and Cellular Functions of Plant RhoGAPs and RhoGDIs. In Integrated G Proteins Signaling in Plants, S. Yalovsky, F. Baluška, and A. Jones, eds (Berlin, Heidelberg: Springer Berlin Heidelberg), pp. 27-48.

Kubo, M., Imai, A., Nishiyama, T., Ishikawa, M., Sato, Y., Kurata, T., Hiwatashi, Y., Reski, R., and Hasebe, M. (2013). System for Stable β-Estradiol-Inducible Gene Expression in the Moss *Physcomitrella patens*. PLoS ONE **8,** e77356. https://doi.org/10.1371/journal.pone.0077356

Kumar, S., and Ruiz, N. (2023). Bacterial AsmA-Like Proteins: Bridging the Gap in Intermembrane Phospholipid Transport. Contact **6**. https://doi.org/10.1177/25152564231185931

Lang, D., Ullrich, K.K., Murat, F., Fuchs, J., Jenkins, J., Haas, F.B., Piednoel, M., Gundlach, H., Van Bel, M., Meyberg, R., Vives, C., Morata, J., Symeonidi, A., Hiss, M., Muchero, W., Kamisugi, Y., Saleh, O., Blanc, G., Decker, E.L., Van Gessel, N., Grimwood, J., Hayes, R.D., Graham, S.W., Gunter, L.E., McDaniel, S.F., Hoernstein, S.N.W., Larsson, A., Li, F.-W., Perroud, P.-F., Phillips, J., Ranjan, P., Rokshar, D.S., Rothfels, C.J., Schneider, L., Shu, S., Stevenson, D.W., Thümmler, F., Tillich, M., Villarreal Aguilar, J.C., Widiez, T., Wong, G.K.-S., Wymore, A., Zhang, Y., Zimmer, A.D., Quatrano, R.S., Mayer, K.F.X., Goodstein, D., Casacuberta, J.M., Vandepoele, K., Reski, R., Cuming, A.C., Tuskan, G.A., Maumus, F., Salse, J., Schmutz, J., and Rensing, S.A. (2018). The *Physcomitrella patens* chromosome-scale assembly reveals moss genome structure and evolution. Plant J. **93,** 515-533. https://doi.org/10.1111/tpj.13801

Lauster, T., Stöckle, D., Gabor, K., Haller, T., Krieger, N., Lotz, P., Mayakrishnan, R., Späth, E., Zimmermann, S., Livanos, P., and Müller, S. (2022). Arabidopsis pavement cell shape formation involves spatially confined ROPGAP regulators. Curr. Biol. **32,** 532-544.e537. https://doi.org/10.1016/j.cub.2021.12.042

Lavy, M., Bloch, D., Hazak, O., Gutman, I., Poraty, L., Sorek, N., Sternberg, H., and Yalovsky, S. (2007). A novel ROP/RAC effector links cell polarity, root-meristem maintenance, and vesicle trafficking. Curr. Biol. **17,** 947-952. https://doi.org/10.1016/j.cub.2007.04.038

Le Bail, A., Scholz, S., and Kost, B. (2013). Evaluation of reference genes for RT qPCR analyses of structure-specific and hormone regulated gene expression in *Physcomitrella patens* gametophytes. PLoS ONE **8,** e70998. https://10.1371/journal.pone.0070998

Le Bail, A., Schulmeister, S., Perroud, P.F., Ntefidou, M., Rensing, S.A., and Kost, B. (2019). Analysis of the localization of fluorescent PpROP1 and PpROP-GEF4 fusion proteins in moss protonemata based on genomic "knock-in" and estradiol-titratable expression. Front. Plant Sci. **10,** 456. https://doi.org/10.3389/fpls.2019.00456

Leebens-Mack, J., Barker, M.S., Carpenter, E.J., and Deyholos, M.K. (2019). One thousand plant transcriptomes and the phylogenomics of green plants. Nature **574,** 679-685. 10.1038/s41586-019-1693-2

Li, C., Sako, Y., Imai, A., Nishiyama, T., Thompson, K., Kubo, M., Hiwatashi, Y., Kabeya, Y., Karlson, D., Wu, S.-H., Ishikawa, M., Murata, T., Benfey, P.N., Sato, Y., Tamada, Y., and Hasebe, M. (2017). A Lin28 homologue reprograms differentiated cells to stem cells in the moss *Physcomitrella patens*. Nat. Commun. **8,** 14242. https://doi.org/10.1038/ncomms14242

Li, H., Wu, G., Ware, D., Davis, K.R., and Yang, Z. (1998). Arabidopsis Rho-Related GTPases: Differential Gene Expression in Pollen and Polar Localization in Fission Yeast1. Plant Physiol. **118,** 407-417. 10.1104/pp.118.2.407

Li, H., Lin, Y., Heath, R.M., Zhu, M.X., and Yang, Z. (1999). Control of pollen tube tip growth by a Rop GTPase-dependent pathway that leads to tip-localized calcium influx. Plant Cell **11,** 1731-1742. 10.1105/tpc.11.9.1731

Liu, L., Niu, L., Ji, K., Wang, Y., Zhang, C., Pan, M., Wang, W., Schiefelbein, J., Yu, F., and An, L. (2023). AXR1modulates trichome morphogenesis through mediating ROP2 stability in Arabidopsis. Plant J. **116,** 756-772. https://doi.org/10.1111/tpj.16403

Luo, N., Yan, A., Liu, G., Guo, J., Rong, D., Kanaoka, M.M., Xiao, Z., Xu, G., Higashiyama, T., Cui, X., and Yang, Z. (2017). Exocytosis-coordinated mechanisms for tip growth underlie pollen tube growth guidance. Nat. Commun. **8,** 1687. https://doi.org/10.1038/s41467-017-01452-0

Molendijk, A.J., Bischoff, F., S.V.Rajendrakumar, C., Friml, J., Braun, M., Gilroy, S., and Palme, K. (2001). *Arabidopsis thaliana* Rop GTPases are localized to tips of root hairs and control polar growth. EMBO J. **20,** 2779-2788. https://doi.org/10.1093/emboj/20.11.2779

Moody, L.A., Kelly, S., Rabbinowitsch, E., and Langdale, J.A. (2018). Genetic Regulation of the 2D to 3D Growth Transition in the Moss *Physcomitrella patens*. Curr. Biol. **28,** 473-478.e475. https://doi.org/10.1016/j.cub.2017.12.052

Müller, S. (2023). Update: on selected ROP cell polarity mechanisms in plant cell morphogenesis. Plant Physiol. **193,** 26-41. 10.1093/plphys/kiad229

Mulvey, H., and Dolan, L. (2023a). RHO of plant signaling was established early in streptophyte evolution. Curr. Biol. **33,** 5515-5525.e5514. https://doi.org/10.1016/j.cub.2023.11.007

Mulvey, H., and Dolan, L. (2023b). RHO GTPase of plants regulates polarized cell growth and cell division orientation during morphogenesis. Curr. Biol. **33,** 2897-2911.e2896. https://doi.org/https://doi.org/10.1016/j.cub.2023.06.015

Nowak, M.A., Boerlijst, M.C., Cooke, J., and Smith, J.M. (1997). Evolution of genetic redundancy. Nature **388,** 167-171. https://doi.org/10.1038/40618

Ntefidou, M., Eklund, D.M., Le Bail, A., Schulmeister, S., Scherbel, F., Brandl, L., Dörfler, W., Eichstädt, C., Bannmüller, A., Ljung, K., and Kost, B. (2023). *Physcomitrium patens* PpRIC, an ancestral CRIB-domain ROP effector, inhibits auxin-induced differentiation of apical initial cells. Cell Rep. **42**. https://doi.org/10.1016/j.celrep.2023.112130

Okano, Y., Aono, N., Hiwatashi, Y., Murata, T., Nishiyama, T., Ishikawa, T., Kubo, M., and Hasebe, M. (2009). A polycomb repressive complex 2 gene regulates apogamy and gives evolutionary insights into early land plant evolution. Proc. Natl. Acad. Sci. U. S. A. **106,** 16321-16326. https://doi.org/10.1073/pnas.0906997106

Ou, H., and Yi, P. (2022). ROP GTPase-dependent polarity establishment during tip growth in plants. New Phytol. **236,** 49-57. https://doi.org/10.1111/nph.18373

Raval, P.K., Ngan, W.Y., Gallie, J., and Agashe, D. (2023). The layered costs and benefits of translational redundancy. eLife **12,** e81005. https://doi.org/10.7554/eLife.81005

Ren, H., Dang, X., Yang, Y., Huang, D., Liu, M., Gao, X., and Lin, D. (2016). SPIKE1 Activates ROP GTPase to Modulate Petal Growth and Shape. Plant Physiol. **172,** 358-371. 10.1104/pp.16.00788

Rensing, S.A., Goffinet, B., Meyberg, R., Wu, S.-Z., and Bezanilla, M. (2020). The Moss *Physcomitrium* (*Physcomitrella*) *patens*: A Model Organism for Non-Seed Plants. Plant Cell **32,** 1361-1376. https://doi.org/10.1105/tpc.19.00828

Rensing, S.A., Ick, J., Fawcett, J.A., Lang, D., Zimmer, A., Van De Peer, Y., and Reski, R. (2007). An ancient genome duplication contributed to the abundance of metabolic genes in the moss *Physcomitrella patens*. BMC Evol. Biol. **7,** 130. https://doi.org/10.1186/1471-2148-7-130

Reski, R., and Abel, W.O. (1985). Induction of budding on chloronemata and caulonemata of the moss, *Physcomitrella patens*, using isopentenyladenine. Planta **165,** 354-358. https://doi.org/10.1007/bf00392232

Roszak, P., Heo, J.-o., Blob, B., Toyokura, K., Sugiyama, Y., de Luis Balaguer, M.A., Lau, W.W.Y., Hamey, F., Cirrone, J., Madej, E., Bouatta, A.M., Wang, X., Guichard, M., Ursache, R., Tavares, H., Verstaen, K., Wendrich, J., Melnyk, C.W., Oda, Y., Shasha, D., Ahnert, S.E., Saeys, Y., De Rybel, B., Heidstra, R., Scheres, B., Grossmann, G., Mähönen, A.P., Denninger, P., Göttgens, B., Sozzani, R., Birnbaum, K.D., and Helariutta, Y. (2021). Cell-by-cell dissection of phloem development links a maturation gradient to cell specialization. Science **374,** eaba5531. https://doi.org/10.1126/science.aba5531

Ruan, J., Lai, L., Ou, H., and Yi, P. (2023). Two subtypes of GTPase-activating proteins coordinate tip
growth and cell size regulation in *Physcomitrium patens*. Nat. Commun. **14,** 7084.
https://doi.org/10.1038/s41467-023-42879-y
Schaefer, D., Zryd, J.-P., Knight, C.D., and Cove, D.J. (1991). Stable transformation of the moss
Physcomitrella patens. Mol. Gen. Genet. **226,** 418-424. https://doi.org/10.1007/BF00260654
Schaefer, D.G., and Zrÿd, J.-P. (1997). Efficient gene targeting in the moss *Physcomitrella patens*. Plant J.
11, 1195-1206. https://doi.org/10.1046/j.1365-313x.1997.11061195.x
Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden,
C., Saalfeld, S., Schmid, B., Tinevez, J.-Y., White, D.J., Hartenstein, V., Eliceiri, K., Tomancak, P., and
Cardona, A. (2012). Fiji: an open-source platform for biological-image analysis. Nat. Methods **9,** 676-
682. https://doi.org/10.1038/nmeth.2019
Schumaker, K.S., and Dietrich, M.A. (1997). Programmed Changes in Form during Moss Development.
Plant Cell **9,** 1099-1107
Stephan, O., Cottier, S., Fahlén, S., Montes-Rodriguez, A., Sun, J., Eklund, D.M., Klahre, U., and Kost, B.
(2014). RISAP Is a TGN-associated RAC5 effector regulating membrane traffic during polar cell growth in
tobacco. Plant Cell **26,** 4426-4447. https://doi.org/10.1105/tpc.114.131078
The UniProt Consortium. (2024). UniProt: the Universal Protein Knowledgebase in 2025. Nucleic Acids
Res.**,** gkae1010. 10.1093/nar/gkae1010
Thelander, M., Olsson, T., and Ronne, H. (2004). Snf1-related protein kinase 1 is needed for growth in a
normal day–night light cycle. EMBO J. **23,** 1900-1910. https://doi.org/10.1038/sj.emboj.7600182
Thelander, M., Nilsson, A., Olsson, T., Johansson, M., Girod, P.-A., Schaefer, D.G., Zrÿd, J.-P., and Ronne,
H. (2007). The moss genes PpSKI1 and PpSKI2 encode nuclear SnRK1 interacting proteins with
homologues in vascular plants. Plant Mol. Biol. **64,** 559-573. https://doi.org/10.1007/s11103-007-9176-
5
Thole, J.M., Perroud, P.F., Quatrano, R.S., and Running, M.P. (2014). Prenylation is required for polar cell
elongation, cell adhesion, and differentiation in Physcomitrella patens. Plant J. **78,** 441-451.
10.1111/tpj.12484
Viaene, T., Landberg, K., Thelander, M., Medvecka, E., Pederson, E., Feraru, E., Cooper, Endymion D.,
Karimi, M., Delwiche, Charles F., Ljung, K., Geisler, M., Sundberg, E., and Friml, J. (2014). Directional
auxin transport mechanisms in early diverging land plants. Curr. Biol. **24,** 2786-2791.
https://doi.org/10.1016/j.cub.2014.09.056
Vidali, L., Augustine, R.C., Kleinman, K.P., and Bezanilla, M. (2007). Profilin is essential for tip growth in
the moss *Physcomitrella patens*. Plant Cell **19,** 3705-3722. https://doi.org/10.1105/tpc.107.053413
Vidali, L., Burkart, G.M., Augustine, R.C., Kerdavid, E., Tüzel, E., and Bezanilla, M. (2010). Myosin XI is
essential for tip growth in *Physcomitrella patens*. Plant Cell **22,** 1868-1882.
https://doi.org/10.1105/tpc.109.073288
Vieten, A., Vanneste, S., Wiśniewska, J., Benková, E., Benjamins, R., Beeckman, T., Luschnig, C., and
Friml, J.I. (2005). Functional redundancy of PIN proteins is accompanied by auxin-dependent cross-
regulation of PIN expression. Development **132,** 4521-4531. https://doi.org/10.1242/dev.02027
Winge, P., Brembu, T., Kristensen, R., and Bones, A.M. (2000). Genetic Structure and Evolution of RAC-
GTPases in Arabidopsis thaliana. Genetics **156,** 1959-1971
Yalovsky, S., Bloch, D., Sorek, N., and Kost, B. (2008). Regulation of membrane trafficking, cytoskeleton
dynamics, and cell polarity by ROP/RAC GTPases. Plant Physiol. **147,** 1527-1543.
10.1104/pp.108.122150
Yi, P., and Goshima, G. (2020). Rho of plants GTPases and cytoskeletal elements control nuclear
positioning and asymmetric cell division during *Physcomitrella patens* branching. Curr. Biol. **30,** 2860-
2868. https://doi.org/10.1016/j.cub.2020.05.022

Figure legends

Figure 1. Knockout of two Pp*ROP*s (*rop*^{2xKO}) promotes caulonema differentiation. Graphs and images based on 5-day-old protonemata or 5-week-old colonies regenerated from protoplasts with the indicated genotypes of double Pp*ROP* knockout mutants and WT using culture conditions listed in Supplementary Table S4. **A)** Average subapical cell length of chloronema and caulonema cells in 5-day-old protonemata. n = 50 cells were analyzed per genotype. The experiment was performed three times with consistent results. **B)** Bright field images of 5-day-old chloronemal and caulonemal filament tips. Asterisks indicate the cell wall between neighboring cells. Scale bars: 25 μm. **C)** Average percentage of caulonema differentiation in 5-day-old protonemal filaments with at least three cells as determined by microscopic observation. n = 60 colonies per genotype were measured in 3 independent experiments. **D-G)** Average size (area) of 5-day-old protonemata **(D)** or 5-week-old colonies **(E)** as determined based on microscopic imaging of chlorophyll autofluorescence **(F**, 5-day-old**)** or pictures recorded using a stereo microscope **(G**, 5-week-old**)**. The mean value of WT was used as a calibrator (relative area = 1). n = 105 colonies per genotype were measured in 3 independent experiments (**D**), or n = 42 colonies per genotype were measured. The experiment was performed three times with consistent results (**E**). Scale bars: 400 μm **(F)**, 10 mm **(G)**. (**A, C-E)** Error bars: standard deviation (SD), dots represent individual data points. Statistical analysis by one-way ANOVA/Tukey's test (pairwise comparisons to WT and relevant pairwise comparisons are displayed, all others see Supplementary Data Set S3): ^{ns} $P > 0.05$ (not significant), * $P ≤ 0.05$, ** $P ≤ 0.01$, *** $P ≤ 0.001$, **** $P ≤ 0.0001$.

Figure 2. Knockout of three Pp*ROP*s (*rop*^{3xKO}) inhibits tip growth of protonemata and rhizoids. Graphs and images based on 5-day-old protonemata or 5-week-old colonies regenerated from protoplasts with the indicated genotypes of triple Pp*ROP* knockout mutants and WT cultivated on media listed in Supplementary Table S4. **A)** Bright field micrographs of 5-day-old chloronemal and caulonemal filament tips. Asterisks indicate the cell wall between neighboring cells. Scale bars: 25 μm. **B)** Average subapical cell length of chloronemal and caulonemal cells in 5-day-old protonemata. n = 50 cells per genotype were measured. The experiment was repeated

three times with consistent results. **C)** Average percentage of caulonema differentiation

in 5-day-old protonemal filaments with at least three cells as determined by microscopic

observation. n = 60 colonies per genotype were measured in 3 independent

experiments. **D, E, G, H)** Average size (area) of 5-day-old protonemata **(D)** or 5-week-

old colonies **(E)** as determined based on microscopic imaging of chlorophyll

autofluorescence **(G)** or pictures recorded using a stereo microscope **(H)**. Scale bars:

400 µm **(G)**, 10 mm **(H)**. The mean value of WT was used as a calibrator (relative area =

1). n = 105 colonies per genotype were measured in 3 independent experiments **(D),** or

n = 45 colonies per genotype were measured. The experiment was repeated three times

with consistent results **(E)**. **F** and **I)** Average length of rhizoids of 5-week-old

gametophores **(F)** based on pictures recorded using a stereo microscope (arrows

indicate the end of rhizoids) **(I)**. Scale bars: 5 mm. n = 21 rhizoids per genotype were

measured. The experiment was repeated three times with consistent results. **B-F)** Error

bars: standard deviation (SD), dots represent individual data points. Statistical analysis

by two-way ANOVA/Tukey's test **(B)** or one-way ANOVA/Tukey's test **(C-F)**

(comparisons to WT are displayed, all others see Supplementary Data Set S3): ^{ns} $P >$

0.05 (not significant), * $P \leq 0.05$, **** $P \leq 0.0001$.

Figure 3. Pp*ROP*s are not altered in their gene expression in rop^{3xKO} mutants and

redundantly complement the rop^{4xKO} mutant. A and **B)** qRT-qPCR of expression

levels of Pp*ROP*s in 1-week-old protonemata cultivated through homogenization

(Supplementary Table S4) with the indicated genotype: rop^{3xKO} mutants **(A)**, rop^{4xKO}

mutant complemented with the coding and 3' UTR sequence of Pp*ROP1* (Pp*ROP4*

identical amino acid sequence) or Pp*ROP2* or Pp*ROP3* downstream of the Pp*ROP1*

promoter (rop^{4xKO}/*ROP1*^{pro}:*ROPX*) **(B)** were compared to WT. Absolute transcript levels

were determined based on standard curves using the value obtained for one WT

replicate of Pp*ROP1* as a calibrator (relative expression = 1). Bars: mean of three

biological and two technical replicates, error bars: standard error of the mean. The

experiment was performed two times with consistent results. **C-E)** Average size (area) of

5-day-old protonemata **(C)** or 4-week-old colonies **(D)** regenerated from protoplasts with

the indicated genotypes: i) WT, ii) *rop3/2/4*, iii) rop^{4xKO}/*ROP1*^{pro}:*ROPX* as determined

based on microscopic imaging of chlorophyll autofluorescence **(E,** 5 days**)** or pictures

recorded using a stereo microscope **(E,** 4 weeks**)**. The mean value of WT was used as a

calibrator (relative area = 1). n = 100 **(C)** or n = 80 **(D)** colonies per genotype were

measured in 3 independent experiments. Error bars: standard deviation (SD), dots

represent individual data points. Scale bars: 250 µm (**E** upper row, 5 days), 5 mm (**E**

lower row, 4 weeks). Statistical analysis by two-way ANOVA/Bonferroni's test **(A)** or one-

way ANOVA/Tukey's test **(B-D)** (pairwise comparisons to WT and relevant pairwise

comparisons are displayed, all others provided in Supplementary Data Set S3): ^{ns} *P* >

0.05 (not significant), * *P* ≤ 0.05, **** *P* ≤ 0.0001.

Figure 4. Loss of all Pp*ROP*s affects cell division and eliminates tip growth.

Analysis of the *rop*^{4xKO} phenotype was performed using protonemata or 5-week-old

colonies cultivated on media listed in Supplementary Table S4 with the indicated

genotypes: i) WT, ii) *rop3/4/1,* iii) *rop3/4/1/2, rop3/2/4/1* (*rop*^{4xKO}), iv) *rop*^{4xKO}/*ind*^{pro}:*ROP2*

uninduced (conditional complementation of *rop*^{4xKO} with the coding and 3' UTR

sequence of Pp*ROP2* downstream of the inducible β-estradiol promoter). **A-C)** 1-week-

old protonemata cultivated through homogenization. **A)** Bright field micrographs of

rop^{4xKO} mutants. Scale bars: 100 µm. **B)** Confocal imaging of the cell division plane

using projections of serial confocal optical sections of protonemata stained with

propidium iodide. Scale bars: 25 µm. **C)** qRT-PCR of relative Pp*ROP2* transcript levels

determined according to the $2^{-\Delta CCT}$ method, using the value obtained for one WT

replicate as a calibrator (relative expression = 1). Bars: mean of three biological and two

technical replicates. The experiment was repeated two times with consistent results. **D-**

G) Analysis of the *rop*^{4xKO} phenotype using 4 or 5-day-old protonemata or 5-week-old

colonies regenerated from protoplasts with the indicated genotypes. *rop*^{4xKO}/*ind*^{pro}:*ROP2*

protoplasts were regenerated without β-estradiol resembling the *rop*^{4xKO} phenotype. **D)**

Bright field images from 5-day-old protonemata (top panel) and images of 5-week-old

colonies (lower panel) were recorded using a stereo microscope. Scale bars: 100 µm

(upper panel), 10 mm (lower panel, black bar), 500 µm (lower panel, white bars). **E)**

Average size (area) of 5-day-old protonemata regenerated from protoplasts as

determined based on microscopic imaging of chlorophyll autofluorescence using the

mean value of WT as calibrator (relative area = 1). n = 50 colonies per genotype

measured in 3 independent experiments. **F** and **G)** Cell division rate in 4-day-old

protonemata was analyzed through projections of serial confocal optical sections

displaying DAPI staining (white) and chlorophyll autofluorescence (red) (scale bars: 100

μm) **(F)** and determination of the average nuclei number **(G)**. n = 50 colonies per

genotype measured in 3 independent experiments. **C, E, G)** Error bars: standard error of

the mean (SEM) **(C)**, standard deviation (SD) **(E** and **G)**, dots represent individual data

points. Statistical analysis by one-way ANOVA/Tukey's test (pairwise comparisons to WT

and relevant pairwise comparisons are displayed, all others see Supplementary Data

Set S3): [ns] $P > 0.05$ (not significant), *** $P ≤ 0.001$, **** $P ≤ 0.0001$.

Figure 5. Titratable Pp*ROP2* expression rescues *rop*^{4xKO}. Protonemata or 4-week-

colonies were regenerated from protoplasts using media solidified with agar except

where otherwise noted (see Supplementary Table S4) with the indicated genotypes: i)

WT, ii) *rop*^{4xKO}/*ind*^{pro}:*ROP2* (conditional complementation of *rop*^{4xKO} with the coding and

3' UTR sequence of Pp*ROP2* downstream of the inducible β-estradiol promoter) treated

with representative β-estradiol concentrations. **A)** Bright field images of 5-day-old

protonemata and images using a stereo microscope of 4-week-old colonies.

rop^{4xKO}/*ind*^{pro}:*ROP2* did not develop gametophores (4 weeks) in the absence or with

minimal PpROP2 expression (0 μM, $2x10^{-5}$ μM β-estradiol). Treatment with $2x10^{-4}$ μM β-

estradiol generated gametophore-like structures only in liquid BCD medium (asterisk)

and at $≥ 4x10^{-4}$ μM β-estradiol gametophores developed on BCD medium solidified with

agar. Closed triangles indicate protonemata, open triangles indicate rhizoids. Scale bars:

200 μm (1st top row), 100 μm (2nd row), 5 mm (3rd row), 1 mm (4th row), 1 mm (5th row,

black bar), 500 μm (5th row, white bars). **B)** Relative transcript levels of Pp*ROP2* were

determined in 7-day-old protonemata (WT, *rop*^{4xKO}/*ind*^{pro}:*ROP2* treated with $9x10^{-4}$ μM β-

estradiol) or 10-day-old protonemata (*rop*^{4xKO}/*ind*^{pro}:*ROP2* treated with $≤ 4x10^{-4}$ μM β-

estradiol) according to the $2^{-ΔΔCt}$ method, using the value obtained for one WT replicate

as calibrator (relative expression = 1). Bars: mean of three biological and two technical

replicates. The experiment was repeated two times with consistent results. **C** and **D)**

Average subapical cell length of chloronemata **(C)** or average percentage of caulonema

differentiation as determined by microscopic observation **(D)** in 5-day-old protonemata. n

= 22 cells **(C)** or n = 30 colonies **(D)** per genotype measured in 3 independent

experiments. **E)** Average size (area) of 5-day-old or 4-week-old colonies using the mean

value of WT as calibrator (relative area = 1). n = 60 colonies per genotype measured in

three independent experiments (5 days). n = 22 colonies per genotype, the experiment

was performed three times with consistent results (4 weeks). **B-E)** Error bars: standard

error of the mean (SEM) **(B)**, standard deviation (SD) **(C-E)**. Dots represent individual

data points. Statistical analysis by one-way ANOVA/Tukey's test (pairwise comparisons

to WT are displayed, all others see Supplementary Data Set S3): ^{ns} P > 0.05 (not

significant), * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

Figure 6. Pp*ROP* overexpression deregulates tip growth and caulonema

differentiation. 1-week old protonemata cultivated through homogenization **(A)** or 5-

day-old protonemata or 4-week colonies regenerated from protoplasts **(B-H)** using

media listed in Supplementary Table S4 with the indicated genotypes: i) WT, ii)

WT/*ind^{pro}:ROP1* (overexpression of the Pp*ROP1* coding sequence and 3' UTR in WT

using the inducible β-estradiol promoter. **A)** Relative transcript levels of Pp*ROP1* were

determined according to the $2^{-\Delta CCT}$ method using the value obtained for one WT

replicate as calibrator (relative expression level = 1). Bars: mean of three biological and

two technical replicates. The experiment was repeated two times with consistent results.

B-D) Average percentage of caulonema differentiation as determined by microscopic

observation **(B)** or average subapical chloronemal cell length **(C)** or cell width **(D)** in

filaments with at least three cells of 5-day-old protonemata. n = 42 cells per genotype

measured in 3 independent experiments. **E)** Bright field micrographs of filament tips of

5-day-old protonemata with the indicated genotypes. Asterisks indicate the cell wall

between neighboring cells, dotted rectangles indicate the magnified apical region. Scale

bars: 25 μm. **F-H)** Average size (area) of 5-day-old protonemata **(F)** or 4-week-old

colonies **(G)** using the mean value of WT as calibrator (relative area = 1) were

determined based on microscopic imaging of chlorophyll autofluorescence **(H**, 1st top

row; 5 days**)** or pictures recorded using a stereo microscope **(H**, 2nd and 3rd rows; 4

weeks**)**. Scale bars: 500 μm (1st top row), 5 mm (2nd row), 1 mm (3rd row). n = 35 **(F)** or n

= 23 **(G)** colonies per genotype. The experiment was repeated two times, and the results

from one representative experiment are shown here. **A-D, F, G)** Error bars: standard

error of the mean (SEM) **(A)**, standard deviation (SD) **(B-D, F, G)**. Dots represent

individual data points. Statistical analysis by one-way ANOVA/Tukey's test (pairwise
comparisons are displayed): ^{ns} $P > 0.05$ (not significant) **** $P \leq 0.0001$.

Figure 7. GTP/GDP cycling is essential for most PpROP functions but not
gametophore initiation. Graphs and images based on protonemata or gametophores
or colonies using culture conditions (Supplementary Table S4) with the indicated
genotypes: i) WT, ii) *rop2/3/4,* iii) *rop*^{4xKO} iv) *rop*^{4xKO}/*ROP1*^{pro}:*ROP1*^{Q64L}
(complementation of *rop*^{4xKO} with the Pp*ROP1* coding and 3' UTR sequence containing
a constitutive active mutation [Q64L] downstream of the endogenous Pp*ROP1*
promoter), v) *rop*^{4xKO}/*ROP1*^{pro}:*ROP1*^{F31L} (complementation of *rop*^{4xKO} with the Pp*ROP1*
coding and the 3' UTR sequence containing a fast cycling mutation [F31L] downstream
of the endogenous Pp*ROP1* promoter). **A)** Relative transcript levels of Pp*ROP1* in 1-
week-old protonemata cultivated through homogenization according to the $2^{-\Delta CCT}$
method, using the value obtained for one WT replicate as a calibrator (relative
expression = 1). Bars: mean of three biological and two technical replicates. The
experiment was repeated two times with consistent results. **B-E)** 5-day-old protonemata
or 5-week-old colonies regenerated from protoplasts (WT, *rop2/3/4,*
rop^{4xKO}/*ROP1*^{pro}:*ROP1*^{F31L}) or cultivated through homogenization (*rop*^{4xKO},
rop^{4xKO}/*ROP1*^{pro}:*ROP1*^{Q64L}). **B)** Images of 5-day-old protonemata recorded using bright
field microscopy (upper row) and images of 5-week-old colonies obtained using a stereo
microscope (WT, *rop2/3/4, rop*^{4xKO}/*ROP1*^{pro}:*ROP1*^{F31L}) or bright field microscopy
(*rop*^{4xKO}, *rop*^{4xKO}/*ROP1*^{pro}:*ROP1*^{Q64L}) (lower row). Scale bars: 100 µm (upper row), 10
mm (black bars, lower row), 400 µm (white bars, lower row). **C)** Images of 5-week-old
gametophores cultivated on solid BCD medium except *rop*^{4xKO}/*ROP1*^{pro}:*ROP1*^{Q64L} that
developed gametophore-like structures only in liquid BCD medium (asterisk) were
recorded using a stereo microscope. Arrow indicates a gametophore-like structure used
for magnification using bright field microscopy (dotted rectangle). Scale bars: 2 mm
(black bars) 200 µm (white bars). **D)** Average size (area) of 5-day-old protonemata or 5-
week-old colonies normalized to the mean value of WT (relative area = 1). n = 30
colonies per genotype; the experiment was repeated three times with consistent results
(5 days) or n = 81 colonies per genotype measured in 3 independent experiments (5
weeks). **E)** Average percentage of caulonema differentiation in 5-day-old protonemal

filaments with at least three cells was determined by microscopic observation. n = 10

colonies per genotype. The experiment was repeated three times with consistent results.

A, **D, E)** Error bars: standard error of the mean (SEM) **(A)**, standard deviation (SD) **(D**

and E), dots represent individual data points. Statistical analysis by one-way

ANOVA/Tukey's test (pairwise comparisons to WT and relevant pairwise comparisons

are displayed, all others see Supplementary Data Set S3). ^{ns} P > 0.05 (not significant), *

P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001.

Figure 8. At*ROP7* or Hs*RHOA* overexpression partially rescues Pp*ROP*s. 1-week-

old protonemata or 4-week-old colonies were cultivated through homogenization using

media described in Supplementary Table S4 with the indicated genotypes: i) WT, ii)

rop1/2/3, iii) *rop*^{4xKO}/*ind*^{pro}:At*ROP7* or *rop*^{4xKO}/*ind*^{pro}:Hs*RHOA* (complementation of

rop^{4xKO} with the coding sequence of At*ROP7* or Hs*RHOA* downstream of the β-estradiol

inducible promoter). **A)** Absolute transcript levels of Pp*ROP4*, At*ROP7,* or Hs*RHOA*

were determined in 1-week-old protonemata with the indicated genotype based on

standard curves, using the value obtained for one WT replicate of Pp*ROP4* as calibrator

(relative expression = 1). Bars: mean of three biological and two technical replicates.

Error bars: standard error of the mean. The experiment was repeated two times with

consistent results. Statistical analysis by one-way ANOVA/Tukey's test (pairwise

comparisons to WT and relevant pairwise comparisons are displayed, all others see

Supplementary Data Set S3): ^{ns} P > 0.05 (not significant), * P ≤ 0.05, ** P ≤ 0.01, *** P ≤

0.001, **** P ≤ 0.0001. **B** and **C)** Bright field micrographs of 1-week-old protonemata **(B)**,

or images of 4-week-old gametophores recorded using a stereo microscope (WT,

rop1/2/3) or gametophore-like structures using a bright field microscope

(*rop*^{4xKO}/*ind*^{pro}:At*ROP7*, *rop*^{4xKO}/*ind*^{pro}:Hs*RHOA*) **(C)**. Scale bars: 50 µm **(B)**, 2 mm (black

bars), 200 µm (white bars) **(C)**.

Figure 9. Overview of PpROP functions. Schematic drawing summarizing PpROP

functions in protonemata and gametophores of *P. patens* with the indicated genotypes: i)

WT (black font), ii) WT/*ind*^{pro}:*ROP1* (Pp*ROP1* overexpresion in WT controlled by the

inducible β-estradiol promoter) (Kubo et al., 2013) (black font), iii) *rop*^{1xKO}, *rop*^{2xKO},

rop^{3xKO}, *rop*^{4xKO} (Pp*ROP* knockout mutants) (red font), iv) *rop*^{4xKO}/*ROP1*^{pro}:*ROP1*^{Q64L},

rop^{4xKO}/*ROP1*^{pro}:*ROP1*^{F31L} (complementation of *rop*^{4xKO} with the coding and 3' UTR

sequence of Pp*ROP1* containing a constitutive active [Q64L] or fast cycling mutation

[F31L] downstream of the Pp*ROP1* promoter) (black font), v) *rop*^4xKO/*ind*^pro:At*ROP7* or

rop^4xKO/*ind*^pro:Hs*RHOA* (complementation of *rop*^4xKO with the coding sequence of

At*ROP7* or Hs*RHOA* downstream of the inducible β-estradiol promoter) (black font), vi)

rop^4xKO/*ind*^pro:*ROP2* (complementation of *rop*^4xKO with the coding and 3' UTR sequence

of Pp*ROP2* downstream of the inducible β-estradiol promoter) (blue font). nd: not

determined.

Supplementary data legends

Supplementary Figure S1. Pp*ROP* gene expression in protonemata (supports **Figs.**

1-5, 7, **Supplementary Fig. S5**). Absolute transcripts levels of Pp*ROP*s in 1-week-old

protonemata of WT cultivated through homogenization on BCDA medium

(Supplementary Table S4) were determined based on standard curves, using the value

obtained for one WT replicate of Pp*ROP1* as calibrator (relative expression = 1). Bars

represent means of three biological and two technical replicates. Error bars: standard

error of the mean (SEM). Statistical analysis by one-way ANOVA/Tukey's test

(Supplementary Data Set S3): ^{ns} P > 0.05 (not significant); * P ≤ 0.05.

Supplementary Figure S2. Editing of the genomic Pp*ROP1* locus through

homologous recombination and summary of transgenic lines (supports **Figs. 1-8,**

Supplementary Figs. S5, S6, S8). **A)** Schematic representation of the Pp*ROP1* genetic

locus and plasmid maps used to generate transgenic lines (Supplementary Table S3),

drawn to scale. pSLU33 was used to generate *rop1* knockout by replacing the genomic

fragment containing all exons and introns of Pp*ROP1* with the expression cassette,

conferring resistance to hygromycin. pFAU508 (*ROP1*^pro:*ROP1*), pFAU509

(*ROP1*^pro:*ROP2*), pFAU517 (*ROP1*^pro:*ROP3*), pFAU306 (*ROP1*^pro:*ROP1*^Q64L) or

pFAU421 (*ROP1*^pro:*ROP1*^F31L) were used to generate *rop*^4xKO complementation lines by

replacing all exons and introns of Pp*ROP1* in *rop3/2/4* with the coding and 3' UTR

sequence of a Pp*ROP* variant. **A** and **D)** Green boxes: exons (numbered) or coding

sequences, gray boxes: UTR sequences, blue boxes: regions used for homologous

recombination targeting, white boxes: resistance markers, arrows: primers used for

genotyping or RT-qPCR (Supplementary Data Set S1), three dots: sequence not

displayed to scale to save space, green numbers: nucleotide position in the Pp*ROP1*

coding region. **B)** Confirmation of transgenic lines through genotyping PCR using genomic DNA with the indicated primers. **C)** Summary of *rop* knockouts indicating the order in which they were generated from left to right (upper table). Summary of *rop*[4xKO] complementation lines generated by replacement of the remaining Pp*ROP* in the indicated *rop*[3xKO] genetic background (red font) with the coding sequence of an *ROP*/*RHO* gene expressed by the endogenous Pp*ROP1* promoter or the inducible β-estradiol-inducible promoter (Kubo et al., 2013) (bottom table). **D)** Vector map of pFAU461 used to overexpress *PpROP1* in WT (WT/*ind*[pro]:*ROP1*) by inserting the coding and 3' UTR sequence of Pp*ROP1* downstream of the inducible β-estradiol-inducible promoter in the PIG1 neutral region through homologous recombination. **E)** Confirmation of WT/*ind*[pro]:*ROP1* transgenic line through genotyping PCR using genomic DNA with the indicated primers.

Supplementary Figure S3. Editing of the genomic Pp*ROP2* and Pp*ROP3* loci through homologous recombination (supports **Figs. 1-5**, **7, Supplementary Figs. S5, S6**). **A** and **C)** Schematic representation of the Pp*ROP2* **(A)** and Pp*ROP3* **(C)** genetic loci and plasmid maps used to generate transgenic lines (Supplementary Table S3), drawn to scale. Green boxes: exons (numbered) or coding sequences, gray boxes: UTR sequences, blue boxes: regions used for homologous recombination targeting, white boxes: resistance markers, arrows: primers used for genotyping or RT-qPCR (Supplementary Data Set S1), three dots: sequence not displayed to scale to save space, green numbers: nucleotide position in the Pp*ROP2* **(A)** or Pp*ROP3* **(C)** coding sequence. **A)** pSLU34 was used to generate *rop2* knockout by replacing the genomic fragment containing all Pp*ROP2* exons and introns with the expression cassette, conferring resistance to zeocin. **C)** pSLU35 was used to generate *rop3* knockout by replacing the genomic fragment containing all Pp*ROP3* exons and introns with the expression cassette, conferring resistance to blasticidin. pFAU431 was used to complement *rop*[4xKO] by replacing all exons and introns of the Pp*ROP3* locus with the coding and 3' UTR sequence of Pp*ROP2* expressed under the control of the β-estradiol-inducible system (Kubo et al., 2013) generating *rop*[4xKO]/*ind*[pro]:*ROP2*. The lexA operator and 35S promoter are depicted, but not the GX8 promoter or XVE regions. **B** and **D)**

Confirmation of transgenic lines through genotyping PCR using genomic DNA with the
indicated primers.

Supplementary Figure S4. Editing of the genomic Pp*ROP4* locus through
homologous recombination (supports **Figs. 1-5**, **7**, **8, Supplementary Figs. S5, S6**).
A) Schematic representation of the Pp*ROP4* genetic locus and plasmid maps used to
generate transgenic lines (Supplementary Table S3), drawn to scale. pSLU36 was used
to generate *rop4* knockout by replacing the genomic fragment containing all Pp*ROP4*
exons and introns with the expression cassette, conferring resistance to G418. pFAU398
(*ind*^pro^:At*ROP7*) or pFAU402 (*ind*^pro^:Hs*RHOA*) were used to complement *rop*^{4xKO} by
replacing all exons and introns of Pp*ROP4* in *rop3/1/2* with the coding sequence of
At*ROP7* or Hs*RHOA* expressed under the control of the β-estradiol-inducible system
(Kubo et al., 2013), generating *rop*^{4xKO}/*ind*^pro^:At*ROP7* or *rop*^{4xKO}/*ind*^pro^:Hs*RHOA*,
respectively. Green boxes: exons (numbered) or coding sequences, gray boxes: UTR
sequences, blue boxes: regions used for homologous recombination targeting, white
boxes: resistance markers, arrows: primers (Supplementary Data Set S1) used for
genotyping or RT-qPCR, three dots: sequence not displayed to scale to save space,
green numbers: nucleotide position in the Pp*ROP4* coding sequence. **B)** Confirmation of
transgenic lines through genotyping PCR using genomic DNA with the indicated primers.
Supplementary Figure S5. **Knockout of a single Pp*ROP* (*rop*^{1xKO}) does not affect**
protonemata (supports **Fig. 1**). **A-F)** Graphs and images based on 5-day-old
protonemata or 5-week-old colonies regenerated from protoplasts were cultivated using
media listed in Supplementary Table S4. **A)** Average subapical cell length of
chloronemata and caulonemata in 5-day-old protonemata. n = 50 cells per genotype.
The experiment was repeated three times with consistent results. **B)** Bright field
micrographs of chloronemal and caulonemal filament tips. Asterisks: cell wall between
apical and subapical cells. Scale bars: 25 μm. **C)** Average percentage of caulonema
differentiation in 5-day-old protonema filaments with at least three cells as determined by
microscopic observation. n = 60 colonies per genotype measured in 3 independent
experiments. **D-F)** Average size of 5-day-old protonemata **(D)** or 5-week-old colonies **(E)**
determined using micrographs of chlorophyll autofluorescence **(F**, upper row**)** or bright
field images **(F**, lower row**)** recorded with a stereo microscope. n = 120 colonies per

genotype measured in 3 independent experiments **(D)** or n = 41 colonies per genotype.
The experiment was performed three times with consistent results **(E)**. Scale bars: 400
µm **(F,** upper row**),** 10 mm **(F,** lower row**)**. **A, C-E)** Error bars: standard deviation (SD),
dots represent individual data points. Statistical analysis by two-way ANOVA/Tukey's
test **(A)** or one-way ANOVA/Tukey's test **(C-E)**. Pairwise comparisons to WT are
displayed, all others see Supplementary Data Set S3: ^{ns} P > 0.05 (not significant); * P ≤
0.05.

Supplementary Figure S6. **PpROPs do not influence the expression of auxin-**
regulated genes or the auxin content (supports **Fig. 2**). **A-C)** Graphs and images
based on 1-week-old protonemata with the indicated genotype cultivated using media
described in Supplementary Table S4. **A)** Free IAA content of WT and rop^{2xKO} lines were
analyzed by gas chromatography-tandem mass spectrometry. Bars: means of n = 11
independent measurements, error bars: standard deviation, dots represent individual
data points. **B)** Confocal microscopy imaging of PpPINA-GFP (Pp$PINA^{pro}$:Pp$PINA$-GFP)
(Viaene et al., 2014) in WT and $rop1/2$. Asterisks indicate the cell wall between the
apical and subapical cell. Scale bars: 25 µm. **C)** RT-qPCR was used to assess the
expression of several auxin-responsive genes related to auxin signaling according to the
$2^{-\Delta\Delta CCT}$ method, using the value obtained for one WT replicate as a calibrator (relative
expression = 1). Bars: mean of three biological and two technical replicates, error bars:
standard error of the mean (SEM). **A** and **C)** Statistical analysis by one-way
ANOVA/Tukey's test (pairwise comparisons to WT are displayed, all others see
Supplementary Data Set S3): ^{ns} P > 0.05 (not significant).

Supplementary Figure S7. β-Estradiol does not influence PpROP expression or
protonemal development (supports **Figs. 5, 6, 8**). Graphs based on protonemata or 4-
week-old colonies of WT cultivated using media listed in Supplementary Table S4. 1 µM
β-estradiol was added to the media from a 10 mM stock solution dissolved in DMSO. **A)**
Relative expression level of Pp$ROP1$ in 1-week-old protonemata cultivated through
homogenization was determined according to the $2^{-\Delta\Delta CCT}$ method, using the value
obtained for one WT replicate as calibrator (relative expression = 1). Bars: mean of three
biological and two technical replicates; error bars: standard error of the mean (SEM).

The experiment was repeated two times with consistent results. **B-F)** Nuclei count and
growth parameters were assessed using protoplasts regenerated for 2 days on PRMB
medium without β-estradiol, followed by 2 days **(B)** or 3 days **(C-E)** on BCDA medium or
4 weeks **(F)** on BCD medium supplemented with 1 μM β-estradiol. **B)** Nuclei were
counted in 4-day-old protonemata stained with DAPI using confocal fluorescence
microscopy. n = 8 colonies per genotype. The experiment was repeated three times with
consistent results. **C** and **D)** Average subapical cell length of chloronema cells **(C)** or
average percentage of caulonema differentiation in 5-day-old protonemal filaments with
at least three cells as determined by microscopic observation **(D)**. n = 42 cells per
genotype were analyzed in 3 independent experiments **(C),** or n = 20 colonies per
genotype were analyzed. The experiment was repeated three times with consistent
results **(D)**. **E** and **F)** Average size (area) of 5-day-old **(E)** or 4-week-old **(F)** colonies
determined based on microscopic imaging of chlorophyll autofluorescence **(E)** or bright
field micrographs **(F)** using a stereo microscope. The mean value of WT was used as a
calibrator (relative area = 1). n = 55 protonemata **(F)** or n = 60 colonies **(E)** per genotype
were analyzed in 3 independent experiments. **A-F)** Error bars: standard error of the
mean (SEM) **(A),** standard deviation (SD) **(C-F)**; dots represent individual data points.
Statistical analysis (Supplementary Data Set S3) by unpaired Student's *t*-test: ^{ns} *P* >
0.05 (not significant).

Supplementary Figure S8. Gametophore-like structures of
rop^{4xKO}/ROP1^{pro}:ROP1^{Q64L} (supports **Fig. 7**). 5-week-old cultures were cultivated in
liquid BCD medium (Supplementary Table S4). Maximum projections of serial optical
sections of cell walls stained with propidium iodide were imaged using confocal
microscopy. Scale bars: 100 μm.
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Figure 1. Knockout of two Pp*ROP*s (*rop*²ˣᴷᴼ) promotes caulonema differentiation. Graphs and images based on 5-day-old protonemata or 5-week-old colonies regenerated from protoplasts with the indicated genotypes of double Pp*ROP* knockout mutants and WT using culture conditions listed in Supplementary Table S4. **A)** Average subapical cell length of chloronema and caulonema cells in 5-day-old protonemata. n = 50 cells were analyzed per genotype. The experiment was performed three times with consistent results. **B)** Bright field images of 5-day-old chloronemal and caulonemal filament tips. Asterisks indicate the cell wall between neighboring cells. Scale bars: 25 μm. **C)** Average percentage of caulonema differentiation in 5-day-old protonemal filaments with at least three cells as determined by microscopic observation. n = 60 colonies per genotype were measured in 3 independent experiments. **D–G)** Average size (area) of 5-day-old protonemata **(D)** or 5-week-old colonies **(E)** as determined based on microscopic imaging of chlorophyll autofluorescence **(F**, 5-day-old**)** or pictures recorded using a stereo microscope **(G**, 5-week-old**)**. The mean value of WT was used as a calibrator (relative area = 1). n = 105 colonies per genotype were measured in 3 independent experiments **(D)**, or n = 42 colonies per genotype were measured. The experiment was performed three times with consistent results **(E)**. Scale bars: 400 μm **(F)**, 10 mm **(G)**. (**A, C–E)** Error bars: standard deviation (SD), dots represent individual data points. Statistical analysis by one-way ANOVA/Tukey's test (pairwise comparisons to WT and relevant pairwise comparisons are displayed, all others see Supplementary Data Set S2): ⁿˢ $P > 0.05$ (not significant), * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

Figure 2. Knockout of three Pp*ROP*s (*rop*³ˣᴷᴼ) inhibits tip growth of protonemata and rhizoids. Graphs and images based on 5-day-old protonemata or 5-week-old colonies regenerated from protoplasts with the indicated genotypes of triple Pp*ROP* knockout mutants and WT cultivated on media listed in Supplementary Table S4. **A)** Bright field micrographs of 5-day-old chloronemal and caulonemal filament tips. Asterisks indicate the cell wall between neighboring cells. Scale bars: 25 μm. **B)** Average subapical cell length of chloronemal and caulonemal cells in 5-day-old protonemata. n = 50 cells per genotype were measured. The experiment was repeated three times with consistent results. **C)** Average percentage of caulonema differentiation in 5-day-old protonemal filaments with at least three cells as determined by microscopic observation. n = 60 colonies per genotype were measured in 3 independent experiments. **D, E, G, H)** Average size (area) of 5-day-old protonemata **(D)** or 5-week-old colonies **(E)** as determined based on microscopic imaging of chlorophyll autofluorescence **(G)** or pictures recorded using a stereo microscope **(H)**. Scale bars: 400 μm **(G)**, 10 mm **(H)**. The mean value of WT was used as a calibrator (relative area = 1). n = 105 colonies per genotype were measured in 3 independent experiments **(D),** or n = 45 colonies per genotype were measured. The experiment was repeated three times with consistent results **(E)**. **F** and **I)** Average length of rhizoids of 5-week-old gametophores **(F)** based on pictures recorded using a stereo microscope (arrows indicate the end of rhizoids) **(I)**. Scale bars: 5 mm. n = 21 rhizoids per genotype were measured. The experiment was repeated three times with consistent results. **B–F)** Error bars: standard deviation (SD), dots represent individual data points. Statistical analysis by two-way ANOVA/Tukey's test **(B)** or one-way ANOVA/Tukey's test **(C–F)** (comparisons to WT are displayed, all others see Supplementary Data Set S2): ⁿˢ $P > 0.05$ (not significant), * $P \leq 0.05$, **** $P \leq 0.0001$.

Figure 3. Pp*ROP*s are not altered in their gene expression in *rop*[3xKO] mutants and redundantly complement the *rop*[4xKO] mutant. A and **B)** qRT-qPCR of expression levels of Pp*ROP*s in 1-week-old protonemata cultivated through homogenization (Supplementary Table S4) with the indicated genotype: *rop*[3xKO] mutants **(A)**, *rop*[4xKO] mutant complemented with the coding and 3' UTR sequence of Pp*ROP1* (Pp*ROP4* identical amino acid sequence) or Pp*ROP2* or Pp*ROP3* downstream of the Pp*ROP1* promoter (*rop*[4xKO]/*ROP1*[pro]:*ROPX*) **(B)** were compared to WT. Absolute transcript levels were determined based on standard curves using the value obtained for one WT replicate of Pp*ROP1* as a calibrator (relative expression = 1). Bars: mean of three biological and two technical replicates, error bars: standard error of the mean. The experiment was performed two times with consistent results. **C–E)** Average size (area) of 5-day-old protonemata **(C)** or 4-week-old colonies **(D)** regenerated from protoplasts with the indicated genotypes: i) WT, ii) *rop3/2/4*, iii) *rop*[4xKO]/*ROP1*[pro]:*ROPX* as determined based on microscopic imaging of chlorophyll autofluorescence **(E,** 5 days**)** or pictures recorded using a stereo microscope **(E,** 4 weeks**)**. The mean value of WT was used as calibrator (relative area = 1). n = 100 **(C)** or n = 80 **(D)** colonies per genotype were measured in 3 independent experiments. Error bars: standard deviation (SD), dots represent individual data points. Scale bars: 250 µm **(E** upper row, 5 days**)**, 5 mm **(E** lower row, 4 weeks**)**. Statistical analysis by two-way ANOVA/Bonferroni's test **(A)** or one-way ANOVA/Tukey's test **(B–D)** (pairwise comparisons to WT and relevant pairwise comparisons are displayed, all others provided in Supplementary Data Set S2): [ns] *P* > 0.05 (not significant), * *P* ≤ 0.05, **** *P* ≤ 0.0001.

Figure 4. Loss of all Pp*ROP*s affects cell division and eliminates tip growth. Analysis of the *rop*[4xKO] phenotype was performed using protonemata or 5-week-old colonies cultivated on media listed in Supplementary Table S4 with the indicated genotypes: i) WT, ii) *rop3/4/1,* iii) *rop3/4/1/2, rop3/2/4/1* (*rop*[4xKO]), iv) *rop*[4xKO]/*ind*[pro]:*ROP2* uninduced (conditional complementation of *rop*[4xKO] with the coding and 3' UTR sequence of Pp*ROP2* downstream of the inducible β-estradiol promoter). **A–C)** 1-week-old protonemata cultivated through homogenization. **A)** Bright field micrographs of *rop*[4xKO] mutants. Scale bars: 100 μm. **B)** Confocal imaging of the cell division plane using projections of serial confocal optical sections of protonemata stained with propidium iodide. Scale bars: 25 μm. **C)** qRT-PCR of relative Pp*ROP2* transcript levels determined according to the $2^{-\Delta\Delta CT}$ method, using the value obtained for one WT replicate as a calibrator (relative expression = 1). Bars: mean of three biological and two technical replicates. The experiment was repeated two times with consistent results. **D–G)** Analysis of the *rop*[4xKO] phenotype using 4 or 5-day-old protonemata or 5-week-old colonies regenerated from protoplasts with the indicated genotypes. *rop*[4xKO]/*ind*[pro]:*ROP2* protoplasts were regenerated without β-estradiol resembling the *rop*[4xKO] phenotype. **D)** Bright field images from 5-day-old protonemata (top panel) and images of 5-week-old colonies (lower panel) were recorded using a stereo microscope. Scale bars: 100 μm (upper panel), 10 mm (lower panel, black bar), 500 μm (lower panel, white bars). **E)** Average size (area) of 5-day-old protonemata regenerated from protoplasts as determined based on microscopic imaging of chlorophyll autofluorescence using the mean value of WT as calibrator (relative area = 1). n = 50 colonies per genotype measured in 3 independent experiments. **F** and **G)** Cell division rate in 4-day-old protonemata was analyzed through projections of serial confocal optical sections displaying DAPI staining (white) and chlorophyll autofluorescence (red) (scale bars: 100 μm) **(F)** and determination of the average nuclei number **(G)**. n = 50 colonies per genotype measured in 3 independent experiments. **C, E, G)** Error bars: standard error of the mean (SEM) **(C)**, standard deviation (SD) **(E** and **G)**, dots represent individual data points. Statistical analysis by one-way ANOVA/Tukey's test (pairwise comparisons to WT and relevant pairwise comparisons are displayed, all others see Supplementary Data Set S2): [ns] $P > 0.05$ (not significant), *** $P \le 0.001$, **** $P \le 0.0001$.

Figure 5. Titratable Pp*ROP2* expression rescues *rop*4xKO. Protonemata or 4-week-colonies were regenerated from protoplasts using media solidified with agar except where otherwise noted (see Supplementary Table S4) with the indicated genotypes: i) WT, ii) *rop*4xKO/*ind*pro:*ROP2* (conditional complementation of *rop*4xKO with the coding and 3' UTR sequence of Pp*ROP2* downstream of the inducible β-estradiol promoter) treated with representative β-estradiol concentrations. **A)** Bright field images of 5-day-old protonemata and images using a stereo microscope of 4-week-old colonies. *rop*4xKO/*ind*pro:*ROP2* did not develop gametophores (4 weeks) in the absence or with minimal PpROP2 expression (0 μM, 2x10⁻⁵ μM β-estradiol). Treatment with 2x10⁻⁴ μM β-estradiol generated gametophore-like structures only in liquid BCD medium (asterisk) and at ≥ 4x10⁻⁴ μM β-estradiol gametophores developed on BCD medium solidified with agar. Closed triangles indicate protonemata, open triangles indicate rhizoids. Scale bars: 200 μm (1st top row), 100 μm (2nd row), 5 mm (3rd row), 1 mm (4th row), 1 mm (5th row, black bar), 500 μm (5th row, white bars). **B)** Relative transcript levels of Pp*ROP2* were determined in 7-day-old protonemata (WT, *rop*4xKO/*ind*pro:*ROP2* treated with 9x10⁻⁴ μM β-estradiol) or 10-day-old protonemata (*rop*4xKO/*ind*pro:*ROP2* treated with ≤ 4x10⁻⁴ μM β-estradiol) according to the $2^{-\Delta\Delta Ct}$ method, using the value obtained for one WT replicate as calibrator (relative expression = 1). Bars: mean of three biological and two technical replicates. The experiment was repeated two times with consistent results. **C** and **D)** Average subapical cell length of chloronemata **(C)** or average percentage of caulonema differentiation as determined by microscopic observation **(D)** in 5-day-old protonemata. n = 22 cells **(C)** or n = 30 colonies **(D)** per genotype measured in 3 independent experiments. **E)** Average size (area) of 5-day-old or 4-week-old colonies using the mean value of WT as calibrator (relative area = 1). n = 60 colonies per genotype measured in three independent experiments (5 days). n = 22 colonies per genotype; the experiment was performed three times with consistent results (4 weeks). **B–E)** Error bars: standard error of the mean (SEM) **(B)**, standard deviation (SD) **(C–E)**. Dots represent individual data points. Statistical analysis by one-way ANOVA/Tukey's test (pairwise comparisons to WT are displayed, all others see Supplementary Data Set S2): ns $P > 0.05$ (not significant), * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

Figure 6. Pp*ROP* overexpression deregulates tip growth and caulonema differentiation. 1-week old protonemata cultivated through homogenization **(A)** or 5-day-old protonemata or 4-week colonies regenerated from protoplasts **(B–H)** using media listed in Supplementary Table S4 with the indicated genotypes: i) WT, ii) WT/*ind*^pro^:*ROP1* (overexpression of the Pp*ROP1* coding sequence and 3' UTR in WT using the inducible β-estradiol promoter. **A)** Relative transcript levels of Pp*ROP1* were determined according to the $2^{-\Delta\Delta CT}$ method using the value obtained for one WT replicate as calibrator (relative expression level = 1). Bars: mean of three biological and two technical replicates. The experiment was repeated two times with consistent results. **B–D)** Average percentage of caulonema differentiation as determined by microscopic observation **(B)** or average subapical chloronemal cell length **(C)** or cell width **(D)** in filaments with at least three cells of 5-day-old protonemata. n = 42 cells per genotype measured in 3 independent experiments. **E)** Bright field micrographs of filament tips of 5-day-old protonemata with the indicated genotypes. Asterisks indicate the cell wall between neighboring cells, dotted rectangles indicate the magnified apical region. Scale bars: 25 μm. **F–H)** Average size (area) of 5-day-old protonemata **(F)** or 4-week-old colonies **(G)** using the mean value of WT as calibrator (relative area = 1) were determined based on microscopic imaging of chlorophyll autofluorescence **(H**, 1st top row; 5 days**)** or pictures recorded using a stereo microscope **(H**, 2nd and 3rd rows; 4 weeks**)**. Scale bars: 500 μm (1st top row), 5 mm (2nd row), 1 mm (3rd row). n = 35 **(F)** or n = 23 **(G)** colonies per genotype. The experiment was repeated two times, and the results from one representative experiment are shown here. **A–D, F, G)** Error bars: standard error of the mean (SEM) **(A)**, standard deviation (SD) **(B–D, F, G)**. Dots represent individual data points. Statistical analysis by one-way ANOVA/Tukey's test (pairwise comparisons are displayed): ^ns^ *P* > 0.05 (not significant) **** *P* ≤ 0.0001..

Figure 7. GTP/GDP cycling is essential for most PpROP functions but not gametophore initiation. Graphs and images based on protonemata or gametophores or colonies using culture conditions (Supplementary Table S4) with the indicated genotypes: i) WT, ii) *rop2/3/4*, iii) *rop*⁴ˣᴷᴼ iv) *rop*⁴ˣᴷᴼ/*ROP1*ᵖʳᵒ:*ROP1*^Q64L (complementation of *rop*⁴ˣᴷᴼ with the Pp*ROP1* coding and 3' UTR sequence containing a constitutive active mutation [Q64L] downstream of the endogenous Pp*ROP1* promoter), v) *rop*⁴ˣᴷᴼ/*ROP1*ᵖʳᵒ:*ROP1*^F31L (complementation of *rop*⁴ˣᴷᴼ with the Pp*ROP1* coding and the 3' UTR sequence containing a fast cycling mutation [F31L] downstream of the endogenous Pp*ROP1* promoter). **A)** Relative transcript levels of Pp*ROP1* in 1-week-old protonemata cultivated through homogenization according to the $2^{-\Delta\Delta CT}$ method, using the value obtained for one WT replicate as a calibrator (relative expression = 1). Bars: mean of three biological and two technical replicates. The experiment was repeated two times with consistent results. **B–E)** 5-day-old protonemata or 5-week-old colonies regenerated from protoplasts (WT, *rop2/3/4*, *rop*⁴ˣᴷᴼ/*ROP1*ᵖʳᵒ:*ROP1*^F31L) or cultivated through homogenization (*rop*⁴ˣᴷᴼ, *rop*⁴ˣᴷᴼ/*ROP1*ᵖʳᵒ:*ROP1*^Q64L). **B)** Images of 5-day-old protonemata recorded using bright field microscopy (upper row) and images of 5-week-old colonies obtained using a stereo microscope (WT, *rop2/3/4*, *rop*⁴ˣᴷᴼ/*ROP1*ᵖʳᵒ:*ROP1*^F31L) or bright field microscopy (*rop*⁴ˣᴷᴼ, *rop*⁴ˣᴷᴼ/*ROP1*ᵖʳᵒ:*ROP1*^Q64L) (lower row). Scale bars: 100 μm (upper row), 10 mm (black bars, lower row), 400 μm (white bars, lower row). **C)** Images of 5-week-old gametophores cultivated on solid BCD medium except *rop*⁴ˣᴷᴼ/*ROP1*ᵖʳᵒ:*ROP1*^Q64L that developed gametophore-like structures only in liquid BCD medium (asterisk) were recorded using a stereo microscope. Arrow indicates a gametophore-like structure used for magnification using bright field microscopy (dotted rectangle). Scale bars: 2 mm (black bars) 200 μm (white bars). **D)** Average size (area) of 5-day-old protonemata or 5-week-old colonies normalized to the mean value of WT (relative area = 1). n = 30 colonies per genotype; the experiment was repeated three times with consistent results (5 days) or n = 81 colonies per genotype measured in 3 independent experiments (5 weeks). **E)** Average percentage of caulonema differentiation in 5-day-old protonemal filaments with at least three cells was determined by microscopic observation. n = 10 colonies per genotype. The experiment was repeated three times with consistent results. **A**, **D, E)** Error bars: standard error of the mean (SEM) **(A)**, standard deviation (SD) **(D** and **E),** dots represent individual data points. Statistical analysis by one-way ANOVA/Tukey's test (pairwise comparisons to WT and relevant pairwise comparisons are displayed, all others see Supplementary Data Set S2). ⁿˢ $P > 0.05$ (not significant), * $P \le 0.05$, ** $P \le 0.01$, *** $P \le 0.001$, **** $P \le 0.0001$.

Figure 8. At*ROP7* or Hs*RHOA* overexpression partially rescues Pp*ROP*s. 1-week-old protonemata or 4-week-old colonies were cultivated through homogenization using media described in Supplementary Table S4 with the indicated genotypes: i) WT, ii) *rop1/2/3*, iii) *rop*^4xKO^/*ind*^pro^:At*ROP7* or *rop*^4xKO^/*ind*^pro^:Hs*RHOA* (complementation of *rop*^4xKO^ with the coding sequence of At*ROP7*, or Hs*RHOA* downstream of the β-estradiol inducible promoter). **A)** Absolute transcript levels of Pp*ROP4*, At*ROP7* or Hs*RHOA* were determined in 1-week-old protonemata with the indicated genotype based on standard curves, using the value obtained for one WT replicate of Pp*ROP4* as calibrator (relative expression = 1). Bars: mean of three biological and two technical replicates. Error bars: standard error of the mean. The experiment was repeated two times with consistent results. Statistical analysis by one-way ANOVA/Tukey's test (pairwise comparisons to WT and relevant pairwise comparisons are displayed, all others see Supplementary Data Set S2): ^{ns} $P > 0.05$ (not significant), * $P ≤ 0.05$, ** $P ≤ 0.01$, *** $P ≤ 0.001$, **** $P ≤ 0.0001$. **B** and **C)** Bright field micrographs of 1-week-old protonemata **(B)** and images of 4-week-old gametophores recorded using a stereo microscope (WT, *rop1/2/3*) or gametophore-like structures using a bright field microscope (*rop*^4xKO^/*ind*^pro^:At*ROP7*, *rop*^4xKO^/*ind*^pro^:Hs*RHOA*) **(C)**. Scale bars: 50 μm **(B)**, 2 mm (black bars), 200 μm (white bars).

Figure 9. Overview of PpROP functions. Schematic drawing summarizing PpROP functions in protonemata and gametophores of *P. patens* with the indicated genotypes: i) WT (black font), ii) WT/*ind*pro:*ROP1* (Pp*ROP1* overexpresion in WT controlled by the inducible β-estradiol promoter) (black font), iii) *rop*1xKO, *rop*2xKO, *rop*3xKO, *rop*4xKO (PpROP knockout mutants) (red font), iv) *rop*4xKO/*ROP1*pro:*ROP1*Q64L, *rop*4xKO/*ROP1*pro:*ROP1*F31L (complementation of *rop*4xKO with the coding and 3' UTR sequence of Pp*ROP1* containing a constitutive active [Q64L] or fast cycling mutation [F31L] downstream of the Pp*ROP1* promoter) (black font), v) *rop*4xKO/*ind*pro:At*ROP7* or *rop*4xKO/*ind*pro:Hs*RHOA* (complementation of *rop*4xKO with the coding sequence of At*ROP7* or Hs*RHOA* downstream of the inducible β-estradiol promoter) (black font), vi) *rop*4xKO/*ind*pro:*ROP2* (complementation of *rop*4xKO with the coding and 3' UTR sequence of Pp*ROP2* downstream of the inducible β-estradiol promoter) (blue font). nd: not determined.

Parsed Citations

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. J. Mol. Biol. 215, 403-410. 10.1016/S0022-2836(05)80360-2
Google Scholar: Author Only Title Only Author and Title

Andersen, S.U., Buechel, S., Zhao, Z., Ljung, K., Novák, O., Busch, W., Schuster, C., and Lohmann, J.U. (2008). Requirement of B2-type cyclin-dependent kinases for meristem integrity in Arabidopsis thaliana. Plant Cell 20, 88-100. https://doi.org/10.1105/tpc.107.054676
Google Scholar: Author Only Title Only Author and Title

Ashton, N.W., and Cove, D.J. (1977). The isolation and preliminary characterisation of auxotrophic and analogue resistant mutants of the moss, Physcomitrella patens. Mol. Gen. Genetics 154, 87-95. https://doi.org/10.1007/BF00265581
Google Scholar: Author Only Title Only Author and Title

Aspenström, P. (2019). The Intrinsic GDP/GTP Exchange Activities of Cdc42 and Rac1 Are Critical Determinants for Their Specific Effects on Mobilization of the Actin Filament System. Cells 8, 759. 10.3390/cells8070759
Google Scholar: Author Only Title Only Author and Title

Aspenström, P. (2022). The Role of Fast-Cycling Atypical RHO GTPases in Cancer. Cancers 14, 1961. https://doi.org/10.3390/cancers14081961
Google Scholar: Author Only Title Only Author and Title

Bao, L., Ren, J., Nguyen, M., Slusarczyk, A.S., Thole, J.M., Martinez, S.P., Huang, J., Fujita, T., and Running, M.P. (2022). The cellular function of ROP GTPase prenylation is important for multicellularity in the moss Physcomitrium patens. Development 149. https://doi.org/10.1242/dev.200279
Google Scholar: Author Only Title Only Author and Title

Berardini, T.Z., Reiser, L., Li, D., Mezheritsky, Y., Muller, R., Strait, E., and Huala, E. (2015). The arabidopsis information resource: Making and mining the "gold standard" annotated reference plant genome. genesis 53, 474-485. https://doi.org/10.1002/dvg.22877
Google Scholar: Author Only Title Only Author and Title

Berken, A., Thomas, C., and Wittinghofer, A. (2005). A new family of RhoGEFs activates the Rop molecular switch in plants. Nature 436, 1176-1180. https://doi.org/10.1038/nature03883
Google Scholar: Author Only Title Only Author and Title

Bi, G., Zhao, S., Yao, J., Wang, H., Zhao, M., Sun, Y., Hou, X., Haas, F.B., Varshney, D., Prigge, M., Rensing, S.A., Jiao, Y., Ma, Y., Yan, J., and Dai, J. (2024). Near telomere-to-telomere genome of the model plant Physcomitrium patens. Nat. Plants 10, 327-343. 10.1038/s41477-023-01614-7
Google Scholar: Author Only Title Only Author and Title

Bishop, A.L., and Hall, A. (2000). Rho GTPases and their effector proteins. Biochem. J. 348, 241-255. 10.1042/bj3480241
Google Scholar: Author Only Title Only Author and Title

Boureux, A., Vignal, E., Faure, S., and Fort, P. (2007). Evolution of the Rho family of ras-like GTPases in eukaryotes. Mol. Biol. Evol. 24, 203-216. 10.1093/molbev/msl145
Google Scholar: Author Only Title Only Author and Title

Brun, F., Gonneau, M., Laloue, M., and Nogué, F. (2003). Identification of Physcomitrella patens genes specific of bud and gametophore formation. Plant Sci. 165, 1267-1274. https://doi.org/10.1016/S0168-9452(03)00335-2
Google Scholar: Author Only Title Only Author and Title

Burkart, G.M., Baskin, T.I., and Bezanilla, M. (2015). A family of ROP proteins that suppress actin dynamics and are essential for polarized growth and cell adhesion. J. Cell Sci. 128, 2553-2564. https://doi.org/10.1242/jcs.172445
Google Scholar: Author Only Title Only Author and Title

Cevik, S., Peng, X., Beyer, T., Pir, M.S., Yenisert, F., Woerz, F., Hoffmann, F., Altunkaynak, B., Pir, B., Boldt, K., Karaman, A., Cakiroglu, M., Oner, S.S., Cao, Y., Ueffing, M., and Kaplan, O.I. (2023). WDR31 displays functional redundancy with GTPase-activating proteins (GAPs) ELMOD and RP2 in regulating IFT complex and recruiting the BBSome to cilium. Life Sci. Alliance 6, e202201844. https://doi.org/10.26508/lsa.202201844
Google Scholar: Author Only Title Only Author and Title

Chen, C.Y.H., Cheung, A.Y., and Wu, H.-M. (2003). Actin-Depolymerizing Factor Mediates Rac/Rop GTPase–Regulated Pollen Tube Growth. Plant Cell 15, 237-249. https://doi.org/10.1105/tpc.007153
Google Scholar: Author Only Title Only Author and Title

Cheng, X., Mwaura, B.W., Chang Stauffer, S.R., and Bezanilla, M. (2020). A fully functional ROP fluorescent fusion protein reveals roles for this GTPase in subcellular and tissue-level patterning. Plant Cell 32, 3436-3451. https://doi.org/10.1105/tpc.20.00440
Google Scholar: Author Only Title Only Author and Title

Clark, K., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J., and Sayers, E.W. (2016). GenBank. Nucleic Acids Res. 44, D67-D72. https://doi.org/10.1093/nar/gkv1276
  Google Scholar: Author Only Title Only Author and Title

Coleman, M.L., Marshall, C.J., and Olson, M.F. (2004). RAS and RHO GTPases in G1-phase cell-cycle regulation. Nat. Rev. Mol. Cell Biol. 5, 355-366. 10.1038/nrm1365
  Google Scholar: Author Only Title Only Author and Title

Collonnier, C., Epert, A, Mara, K., Maclot, F., Guyon-Debast, A, Charlot, F., White, C., Schaefer, D.G., and Nogué, F. (2017). CRISPR-Cas9-mediated efficient directed mutagenesis and RAD51-dependent and RAD51-independent gene targeting in the moss Physcomitrella patens. Plant Biotechnol. J. 15, 122-131. 10.1111/pbi.12596
  Google Scholar: Author Only Title Only Author and Title

Cove, D. (2005). The Moss Physcomitrella patens. Annu. Rev. Genet. 39, 339 - 358. 10.1146/annurev.genet.39.073003.110214
  Google Scholar: Author Only Title Only Author and Title

Cove, D., and Knight, C. (1993). The moss Physcomitrella patens, a model system with potential for the study of plant reproduction. Plant Cell 5, 1483-1488. https://doi.org/10.1105/tpc.5.10.1483
  Google Scholar: Author Only Title Only Author and Title

Cove, D.J., Perroud, P.-F., Charron, A.J., McDaniel, S.F., Khandelwal, A, and Quatrano, R.S. (2009). Isolation and Regeneration of Protoplasts of the Moss Physcomitrella patens. Cold Spring Harbor Protocols 2009. https://doi.org/doi:10.1101/pdb.prot5140
  Google Scholar: Author Only Title Only Author and Title

Dean, E.J., Davis, J.C., Davis, R.W., and Petrov, D.A. (2008). Pervasive and Persistent Redundancy among Duplicated Genes in Yeast. PLOS Genet. 4, e1000113. https://doi.org/10.1371/journal.pgen.1000113
  Google Scholar: Author Only Title Only Author and Title

Denninger, P., Reichelt, A, Schmidt, V.A.F., Mehlhorn, D.G., Asseck, L.Y., Stanley, C.E., Keinath, N.F., Evers, J.-F., Grefen, C., and Grossmann, G. (2019). Distinct RopGEFs Successively Drive Polarization and Outgrowth of Root Hairs. Curr. Biol. 29, 1854-1865. https://doi.org/10.1016/j.cub.2019.04.059
  Google Scholar: Author Only Title Only Author and Title

Eklund, D.M., Svensson, E.M., and Kost, B. (2010). Physcomitrella patens: a model to investigate the role of RAC/ROP GTPase signalling in tip growth. J. Exp. Bot. 61, 1917-1937. https://doi.org/10.1093/jxb/erq080
  Google Scholar: Author Only Title Only Author and Title

Etienne-Manneville, S., and Hall, A. (2002). Rho GTPases in cell biology. Nature 420, 629-635. https://doi.org/10.1038/nature01148
  Google Scholar: Author Only Title Only Author and Title

Fantes, P., and Nurse, P. (1977). Control of cell size at division in fission yeast by a growth-modulated size control over nuclear division. Exp. Cell Res. 107, 377-386. https://doi.org/10.1016/0014-4827(77)90359-7
  Google Scholar: Author Only Title Only Author and Title

Feiguelman, G., Fu, Y., and Yalovsky, S. (2018). ROP GTPases Structure-Function and Signaling Pathways. Plant Physiol. 176, 57-79. https://doi.org/10.1104/pp.17.01415
  Google Scholar: Author Only Title Only Author and Title

Fu, Y., Gu, Y., Zheng, Z, Wasteneys, G., and Yang, Z. (2005). Arabidopsis interdigitating cell growth requires two antagonistic pathways with opposing action on cell morphogenesis. Cell 120, 687-700. 10.1016/j.cell.2004.12.026
  Google Scholar: Author Only Title Only Author and Title

Gao, B., Chen, M.-X., Li, X.-S., Liang, Y.-Q., Zhang, D.-Y., Wood, A.J., Oliver, M.J., and Zhang, J.-H. (2022). Ancestral gene duplications in mosses characterized by integrated phylogenomic analyses. J. Syst. Evol. 60, 144-159. https://doi.org/10.1111/jse.12683
  Google Scholar: Author Only Title Only Author and Title

Goodstein, D.M., Shu, S., Howson, R., Neupane, R., Hayes, R.D., Fazo, J., Mitros, T., Dirks, W., Hellsten, U., Putnam, N., and Rokhsar, D.S. (2012). Phytozome: a comparative platform for green plant genomics. Nucleic Acids Res. 40, D1178-D1186. https://doi.org/10.1093/nar/gkr944
  Google Scholar: Author Only Title Only Author and Title

Green, M.R., and Sambrook, J. (2012). Molecular cloning: A laboratory manual. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).
  Google Scholar: Author Only Title Only Author and Title

Grimsley, N.H., Ashton, N.W., and Cove, D.J. (1977). The production of somatic hybrids by protoplast fusion in the moss, Physcomitrella patens. Mol. Gen. Genet. 154, 97-100. https://doi.org/10.1007/bf00265582
  Google Scholar: Author Only Title Only Author and Title

Gu, Y., Vernoud, V., Fu, Y., and Yang, Z (2003a). ROP GTPase regulation of pollen tube growth through the dynamics of tip-localized F-actin. J. Exp. Bot. 54, 93-101. 10.1093/jxb/erg035
Google Scholar: Author Only Author and Title

Gu, Z, Steinmetz, L.M., Gu, X., Scharfe, C., Davis, R.W., and Li, W.-H. (2003b). Role of duplicate genes in genetic robustness against null mutations. Nature 421, 63-66. https://doi.org/10.1038/nature01198
Google Scholar: Author Only Author and Title

Hall, A, and Lalli, G. (2010). Rho and Ras GTPases in axon growth, guidance, and branching. Cold Spring Harb Perspect Biol 2, a001818. https://doi.org/10.1101/cshperspect.a001818
Google Scholar: Author Only Title Only Author and Title

Harrison, C.J., Roeder, A.H.K., Meyerowitz, E.M., and Langdale, J.A (2009). Local Cues and Asymmetric Cell Divisions Underpin Body Plan Transitions in the Moss Physcomitrella patens. Curr. Biol. 19, 461-471. https://doi.org/10.1016/j.cub.2009.02.050
Google Scholar: Author Only Title Only Author and Title

Heasman, S.J., and Ridley, A.J. (2008). Mammalian Rho GTPases: new insights into their functions from in vivo studies. Nat. Rev. Mol. Cell Biol. 9, 690-701. 10.1038/nrm2476
Google Scholar: Author Only Title Only Author and Title

Hiwatashi, Y., Obara, M., Sato, Y., Fujita, T., Murata, T., and Hasebe, M. (2008). Kinesins are indispensable for interdigitation of phragmoplast microtubules in the moss Physcomitrella patens. Plant Cell 20, 3094-3106. https://doi.org/10.1105/tpc.108.061705
Google Scholar: Author Only Title Only Author and Title

Hodge, R.G., and Ridley, A.J. (2016). Regulating Rho GTPases and their regulators. Nat. Rev. Mol. Cell Biol. 17, 496-510. https://doi.org/10.1038/nrm.2016.67
Google Scholar: Author Only Title Only Author and Title

Humphries, J.A, Vejlupkova, Z, Luo, A, Meeley, R.B., Sylvester, A.W., Fowler, J.E., and Smith, L.G. (2011). ROP GTPases Act with the Receptor-Like Protein PAN1 to Polarize Asymmetric Cell Division in Maize. Plant Cell 23, 2273-2284. https//doi.org.10.1105/tpc.111.085597
Google Scholar: Author Only Title Only Author and Title

Ito, K., Ren, J., and Fujita, T. (2014). Conserved function of Rho-related Rop/RAC GTPase signaling in regulation of cell polarity in Physcomitrella patens. Gene 544, 241-247. https://doi.org/10.1016/j.gene.2014.04.057
Google Scholar: Author Only Title Only Author and Title

Jaeger, R., and Moody, L.A (2021). A fundamental developmental transition in Physcomitrium patens is regulated by evolutionarily conserved mechanisms. Evol. Devel. 23, 123-136. https://doi.org/10.1111/ede.12376
Google Scholar: Author Only Title Only Author and Title

Jang, G., and Dolan, L. (2011). Auxin promotes the transition from chloronema to caulonema in moss protonema by positively regulating PpRSL1and PpRSL2 in Physcomitrella patens. New Phytol. 192, 319-327. https://doi.org/10.1111/j.1469-8137.2011.03805.x
Google Scholar: Author Only Title Only Author and Title

Jones, A.R., Forero-Vargas, M., Withers, S.P., Smith, R.S., Traas, J., Dewitte, W., and Murray, J.A.H. (2017). Cell-size dependent progression of the cell cycle creates homeostasis and flexibility of plant cell size. Nat. Commun. 8, 15060. https://doi.org/10.1038/ncomms15060
Google Scholar: Author Only Title Only Author and Title

Katzen, F. (2007). Gateway® recombinational cloning: a biological operating system. Expert Opin. Drug Discov. 2, 571-589. https://doi.org/10.1517/17460441.2.4.571
Google Scholar: Author Only Title Only Author and Title

Kawano, Y., Kaneko-Kawano, T., and Shimamoto, K. (2014). Rho family GTPase-dependent immunity in plants and animals. Front. Plant Sci. 5. https://doi.org/10.3389/fpls.2014.00522
Google Scholar: Author Only Title Only Author and Title

Klahre, U., and Kost, B. (2006). Tobacco RhoGTPase ACTIVATING PROTEIN1 Spatially Restricts Signaling of RAC/Rop to the Apex of Pollen Tubes. Plant Cell 18, 3033-3046. https://doi.org/10.1105/tpc.106.045336
Google Scholar: Author Only Title Only Author and Title

Kofuji, R., and Hasebe, M. (2014). Eight types of stem cells in the life cycle of the moss Physcomitrella patens. Curr. Opin. Plant Biol. 17, 13-21. 10.1016/j.pbi.2013.10.007
Google Scholar: Author Only Title Only Author and Title

Kondrashov, F.A, Rogozin, I.B., Wolf, Y.I., and Koonin, E.V. (2002). Selection in the evolution of gene duplications. Genome Biol. 3. https://doi.org/10.1186/gb-2002-3-2-research0008
Google Scholar: Author Only Author and Title

Kost, B. (2008). Spatial control of Rho (Rac-Rop) signaling in tip-growing plant cells. Trends Cell Biol. 18, 119-127. 10.1016/j.tcb.2008.01.003
  Google Scholar: Author Only Title Only Author and Title

Kost, B. (2010). Regulatory and Cellular Functions of Plant RhoGAPs and RhoGDIs. In Integrated G Proteins Signaling in Plants, S. Yalovsky, F. Baluška, and A. Jones, eds (Berlin, Heidelberg: Springer Berlin Heidelberg), pp. 27-48.
  Google Scholar: Author Only Title Only Author and Title

Kubo, M., Imai, A., Nishiyama, T., Ishikawa, M., Sato, Y., Kurata, T., Hiwatashi, Y., Reski, R., and Hasebe, M. (2013). System for Stable β-Estradiol-Inducible Gene Expression in the Moss Physcomitrella patens. PLoS ONE 8, e77356. https://doi.org/10.1371/journal.pone.0077356
  Google Scholar: Author Only Title Only Author and Title

Kumar, S., and Ruiz, N. (2023). Bacterial AsmA-Like Proteins: Bridging the Gap in Intermembrane Phospholipid Transport. Contact 6. https://doi.org/10.1177/25152564231185931
  Google Scholar: Author Only Title Only Author and Title

Lang, D., Ullrich, K.K., Murat, F., Fuchs, J., Jenkins, J., Haas, F.B., Piednoel, M., Gundlach, H., Van Bel, M., Meyberg, R., Vives, C., Morata, J., Symeonidi, A., Hiss, M., Muchero, W., Kamisugi, Y., Saleh, O., Blanc, G., Decker, E.L., Van Gessel, N., Grimwood, J., Hayes, R.D., Graham, S.W., Gunter, L.E., McDaniel, S.F., Hoernstein, S.N.W., Larsson, A., Li, F.-W., Perroud, P.-F., Phillips, J., Ranjan, P., Rokshar, D.S., Rothfels, C.J., Schneider, L., Shu, S., Stevenson, D.W., Thümmler, F., Tillich, M., Villarreal Aguilar, J.C., Widiez, T., Wong, G.K.-S., Wymore, A., Zhang, Y., Zimmer, A.D., Quatrano, R.S., Mayer, K.F.X., Goodstein, D., Casacuberta, J.M., Vandepoele, K., Reski, R., Cuming, A.C., Tuskan, G.A., Maumus, F., Salse, J., Schmutz, J., and Rensing, S.A. (2018). The Physcomitrella patens chromosome-scale assembly reveals moss genome structure and evolution. Plant J. 93, 515-533. https://doi.org/10.1111/tpj.13801
  Google Scholar: Author Only Title Only Author and Title

Lauster, T., Stöckle, D., Gabor, K., Haller, T., Krieger, N., Lotz, P., Mayakrishnan, R., Späth, E., Zimmermann, S., Livanos, P., and Müller, S. (2022). Arabidopsis pavement cell shape formation involves spatially confined ROPGAP regulators. Curr. Biol. 32, 532-544.e537. https://doi.org/10.1016/j.cub.2021.12.042
  Google Scholar: Author Only Title Only Author and Title

Lavy, M., Bloch, D., Hazak, O., Gutman, I., Poraty, L., Sorek, N., Sternberg, H., and Yalovsky, S. (2007). A novel ROP/RAC effector links cell polarity, root-meristem maintenance, and vesicle trafficking. Curr. Biol. 17, 947-952. https://doi.org/10.1016/j.cub.2007.04.038
  Google Scholar: Author Only Title Only Author and Title

Le Bail, A., Scholz, S., and Kost, B. (2013). Evaluation of reference genes for RT qPCR analyses of structure-specific and hormone regulated gene expression in Physcomitrella patens gametophytes. PLoS ONE 8, e70998. https://10.1371/journal.pone.0070998
  Google Scholar: Author Only Title Only Author and Title

Le Bail, A., Schulmeister, S., Perroud, P.F., Ntefidou, M., Rensing, S.A., and Kost, B. (2019). Analysis of the localization of fluorescent PpROP1 and PpROP-GEF4 fusion proteins in moss protonemata based on genomic "knock-in" and estradiol-titratable expression. Front. Plant Sci. 10, 456. https://doi.org/10.3389/fpls.2019.00456
  Google Scholar: Author Only Title Only Author and Title

Leebens-Mack, J., Barker, M.S., Carpenter, E.J., and Deyholos, M.K. (2019). One thousand plant transcriptomes and the phylogenomics of green plants. Nature 574, 679-685. 10.1038/s41586-019-1693-2
  Google Scholar: Author Only Title Only Author and Title

Li, C., Sako, Y., Imai, A., Nishiyama, T., Thompson, K., Kubo, M., Hiwatashi, Y., Kabeya, Y., Karlson, D., Wu, S.-H., Ishikawa, M., Murata, T., Benfey, P.N., Sato, Y., Tamada, Y., and Hasebe, M. (2017). A Lin28 homologue reprograms differentiated cells to stem cells in the moss Physcomitrella patens. Nat. Commun. 8, 14242. https://doi.org/10.1038/ncomms14242
  Google Scholar: Author Only Title Only Author and Title

Li, H., Wu, G., Ware, D., Davis, K.R., and Yang, Z. (1998). Arabidopsis Rho-Related GTPases: Differential Gene Expression in Pollen and Polar Localization in Fission Yeast1. Plant Physiol. 118, 407-417. 10.1104/pp.118.2.407
  Google Scholar: Author Only Title Only Author and Title

Li, H., Lin, Y., Heath, R.M., Zhu, M.X., and Yang, Z. (1999). Control of pollen tube tip growth by a Rop GTPase-dependent pathway that leads to tip-localized calcium influx. Plant Cell 11, 1731-1742. 10.1105/tpc.11.9.1731
  Google Scholar: Author Only Title Only Author and Title

Liu, L., Niu, L., Ji, K., Wang, Y., Zhang, C., Pan, M., Wang, W., Schiefelbein, J., Yu, F., and An, L. (2023). AXR1 modulates trichome morphogenesis through mediating ROP2 stability in Arabidopsis. Plant J. 116, 756-772. https://doi.org/10.1111/tpj.16403
  Google Scholar: Author Only Title Only Author and Title

Luo, N., Yan, A., Liu, G., Guo, J., Rong, D., Kanaoka, M.M., Xiao, Z., Xu, G., Higashiyama, T., Cui, X., and Yang, Z. (2017). Exocytosis-coordinated mechanisms for tip growth underlie pollen tube growth guidance. Nat. Commun. 8, 1687.

https://doi.org/10.1038/s41467-017-01452-0
Google Scholar: Author Only Title Only Author and Title

Molendijk, A.J., Bischoff, F., S.V.Rajendrakumar, C., Friml, J., Braun, M., Gilroy, S., and Palme, K. (2001). Arabidopsis thaliana Rop GTPases are localized to tips of root hairs and control polar growth. EMBO J. 20, 2779-2788. https://doi.org/10.1093/emboj/20.11.2779
Google Scholar: Author Only Title Only Author and Title

Moody, L.A., Kelly, S., Rabbinowitsch, E., and Langdale, J.A. (2018). Genetic Regulation of the 2D to 3D Growth Transition in the Moss Physcomitrella patens. Curr. Biol. 28, 473-478.e475. https://doi.org/10.1016/j.cub.2017.12.052
Google Scholar: Author Only Title Only Author and Title

Müller, S. (2023). Update: on selected ROP cell polarity mechanisms in plant cell morphogenesis. Plant Physiol. 193, 26-41. 10.1093/plphys/kiad229
Google Scholar: Author Only Title Only Author and Title

Mulvey, H., and Dolan, L. (2023a). RHO of plant signaling was established early in streptophyte evolution. Curr. Biol. 33, 5515-5525.e5514. https://doi.org/10.1016/j.cub.2023.11.007
Google Scholar: Author Only Title Only Author and Title

Mulvey, H., and Dolan, L. (2023b). RHO GTPase of plants regulates polarized cell growth and cell division orientation during morphogenesis. Curr. Biol. 33, 2897-2911.e2896. https://doi.org/https://doi.org/10.1016/j.cub.2023.06.015
Google Scholar: Author Only Title Only Author and Title

Nowak, M.A., Boerlijst, M.C., Cooke, J., and Smith, J.M. (1997). Evolution of genetic redundancy. Nature 388, 167-171. https://doi.org/10.1038/40618
Google Scholar: Author Only Title Only Author and Title

Ntefidou, M., Eklund, D.M., Le Bail, A., Schulmeister, S., Scherbel, F., Brandl, L., Dörfler, W., Eichstädt, C., Bannmüller, A., Ljung, K., and Kost, B. (2023). Physcomitrium patens PpRIC, an ancestral CRIB-domain ROP effector, inhibits auxin-induced differentiation of apical initial cells. Cell Rep. 42. https://doi.org/10.1016/j.celrep.2023.112130
Google Scholar: Author Only Title Only Author and Title

Okano, Y., Aono, N., Hiwatashi, Y., Murata, T., Nishiyama, T., Ishikawa, T., Kubo, M., and Hasebe, M. (2009). A polycomb repressive complex 2 gene regulates apogamy and gives evolutionary insights into early land plant evolution. Proc. Natl. Acad. Sci. U. S. A. 106, 16321-16326. https://doi.org/10.1073/pnas.0906997106
Google Scholar: Author Only Title Only Author and Title

Ou, H., and Yi, P. (2022). ROP GTPase-dependent polarity establishment during tip growth in plants. New Phytol. 236, 49-57. https://doi.org/10.1111/nph.18373
Google Scholar: Author Only Title Only Author and Title

Raval, P.K., Ngan, W.Y., Gallie, J., and Agashe, D. (2023). The layered costs and benefits of translational redundancy. eLife 12, e81005. https://doi.org/10.7554/eLife.81005
Google Scholar: Author Only Title Only Author and Title

Ren, H., Dang, X., Yang, Y., Huang, D., Liu, M., Gao, X., and Lin, D. (2016). SPIKE1 Activates ROP GTPase to Modulate Petal Growth and Shape. Plant Physiol. 172, 358-371. 10.1104/pp.16.00788
Google Scholar: Author Only Title Only Author and Title

Rensing, S.A., Goffinet, B., Meyberg, R., Wu, S.-Z., and Bezanilla, M. (2020). The Moss Physcomitrium (Physcomitrella) patens: A Model Organism for Non-Seed Plants. Plant Cell 32, 1361-1376. https://doi.org/10.1105/tpc.19.00828
Google Scholar: Author Only Title Only Author and Title

Rensing, S.A., Ick, J., Fawcett, J.A., Lang, D., Zimmer, A., Van De Peer, Y., and Reski, R. (2007). An ancient genome duplication contributed to the abundance of metabolic genes in the moss Physcomitrella patens. BMC Evol. Biol. 7, 130. https://doi.org/10.1186/1471-2148-7-130
Google Scholar: Author Only Title Only Author and Title

Reski, R., and Abel, W.O. (1985). Induction of budding on chloronemata and caulonemata of the moss, Physcomitrella patens, using isopentenyladenine. Planta 165, 354-358. https://doi.org/10.1007/bf00392232
Google Scholar: Author Only Title Only Author and Title

Roszak, P., Heo, J.-o., Blob, B., Toyokura, K., Sugiyama, Y., de Luis Balaguer, M.A., Lau, W.W.Y., Hamey, F., Cirrone, J., Madej, E., Bouatta, A.M., Wang, X., Guichard, M., Ursache, R., Tavares, H., Verstaen, K., Wendrich, J., Melnyk, C.W., Oda, Y., Shasha, D., Ahnert, S.E., Saeys, Y., De Rybel, B., Heidstra, R., Scheres, B., Grossmann, G., Mähönen, A.P., Denninger, P., Göttgens, B., Sozzani, R., Birnbaum, K.D., and Helariutta, Y. (2021). Cell-by-cell dissection of phloem development links a maturation gradient to cell specialization. Science 374, eaba5531. https://doi.org/10.1126/science.aba5531
Google Scholar: Author Only Title Only Author and Title

Ruan, J., Lai, L., Ou, H., and Yi, P. (2023). Two subtypes of GTPase-activating proteins coordinate tip growth and cell size regulation in Physcomitrium patens. Nat. Commun. 14, 7084. https://doi.org/10.1038/s41467-023-42879-y
Google Scholar: Author Only Title Only Author and Title

Schaefer, D., Zryd, J.-P., Knight, C.D., and Cove, D.J. (1991). Stable transformation of the moss Physcomitrella patens. Mol. Gen. Genet. 226, 418-424. https://doi.org/10.1007/BF00260654
Google Scholar: Author Only Title Only Author and Title

Schaefer, D.G., and Zrÿd, J.-P. (1997). Efficient gene targeting in the moss Physcomitrella patens. Plant J. 11, 1195-1206. https://doi.org/10.1046/j.1365-313x.1997.11061195.x
Google Scholar: Author Only Title Only Author and Title

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.-Y., White, D.J., Hartenstein, V., Eliceiri, K., Tomancak, P., and Cardona, A. (2012). Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676-682. https://doi.org/10.1038/nmeth.2019
Google Scholar: Author Only Title Only Author and Title

Schumaker, K.S., and Dietrich, M.A. (1997). Programmed Changes in Form during Moss Development. Plant Cell 9, 1099-1107
Google Scholar: Author Only Title Only Author and Title

Stephan, O., Cottier, S., Fahlén, S., Montes-Rodriguez, A., Sun, J., Eklund, D.M., Klahre, U., and Kost, B. (2014). RISAP Is a TGN-associated RAC5 effector regulating membrane traffic during polar cell growth in tobacco. Plant Cell 26, 4426-4447. https://doi.org/10.1105/tpc.114.131078
Google Scholar: Author Only Title Only Author and Title

The UniProt Consortium. (2024). UniProt: the Universal Protein Knowledgebase in 2025. Nucleic Acids Res., gkae1010. 10.1093/nar/gkae1010
Google Scholar: Author Only Title Only Author and Title

Thelander, M., Olsson, T., and Ronne, H. (2004). Snf1-related protein kinase 1 is needed for growth in a normal day–night light cycle. EMBO J. 23, 1900-1910. https://doi.org/10.1038/sj.emboj.7600182
Google Scholar: Author Only Title Only Author and Title

Thelander, M., Nilsson, A., Olsson, T., Johansson, M., Girod, P.-A., Schaefer, D.G., Zrÿd, J.-P., and Ronne, H. (2007). The moss genes PpSKI1 and PpSKI2 encode nuclear SnRK1 interacting proteins with homologues in vascular plants. Plant Mol. Biol. 64, 559-573. https://doi.org/10.1007/s11103-007-9176-5
Google Scholar: Author Only Title Only Author and Title

Thole, J.M., Perroud, P.F., Quatrano, R.S., and Running, M.P. (2014). Prenylation is required for polar cell elongation, cell adhesion, and differentiation in Physcomitrella patens. Plant J. 78, 441-451. 10.1111/tpj.12484
Google Scholar: Author Only Title Only Author and Title

Viaene, T., Landberg, K., Thelander, M., Medvecka, E., Pederson, E., Feraru, E., Cooper, Endymion D., Karimi, M., Delwiche, Charles F., Ljung, K., Geisler, M., Sundberg, E., and Friml, J. (2014). Directional auxin transport mechanisms in early diverging land plants. Curr. Biol. 24, 2786-2791. https://doi.org/10.1016/j.cub.2014.09.056
Google Scholar: Author Only Title Only Author and Title

Vidali, L., Augustine, R.C., Kleinman, K.P., and Bezanilla, M. (2007). Profilin is essential for tip growth in the moss Physcomitrella patens. Plant Cell 19, 3705-3722. https://doi.org/10.1105/tpc.107.053413
Google Scholar: Author Only Title Only Author and Title

Vidali, L., Burkart, G.M., Augustine, R.C., Kerdavid, E., Tüzel, E., and Bezanilla, M. (2010). Myosin XI is essential for tip growth in Physcomitrella patens. Plant Cell 22, 1868-1882. https://doi.org/10.1105/tpc.109.073288
Google Scholar: Author Only Title Only Author and Title

Vieten, A., Vanneste, S., WiśNiewska, J., Benková, E., Benjamins, R., Beeckman, T., Luschnig, C., and Friml, J.I. (2005). Functional redundancy of PIN proteins is accompanied by auxin-dependent cross-regulation of PIN expression. Development 132, 4521-4531. https://doi.org/10.1242/dev.02027
Google Scholar: Author Only Title Only Author and Title

Winge, P., Brembu, T., Kristensen, R., and Bones, A.M. (2000). Genetic Structure and Evolution of RAC-GTPases in Arabidopsis thaliana. Genetics 156, 1959-1971
Google Scholar: Author Only Title Only Author and Title

Yalovsky, S., Bloch, D., Sorek, N., and Kost, B. (2008). Regulation of membrane trafficking, cytoskeleton dynamics, and cell polarity by ROP/RAC GTPases. Plant Physiol. 147, 1527-1543. 10.1104/pp.108.122150
Google Scholar: Author Only Title Only Author and Title

Yi, P., and Goshima, G. (2020). Rho of plants GTPases and cytoskeletal elements control nuclear positioning and asymmetric cell division during Physcomitrella patens branching. Curr. Biol. 30, 2860-2868. https://doi.org/10.1016/j.cub.2020.05.022