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Report

A ROP GTPase-Dependent Auxin Signaling Pathway Regulates the Subcellular Distribution of PIN2 in *Arabidopsis* Roots

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Summary

PIN-FORMED (PIN) protein-mediated auxin polar transport is critically important for development, pattern formation, and morphogenesis in plants. Auxin has been implicated in the regulation of polar auxin transport by inhibiting PIN endocytosis [1, 2], but how auxin regulates this process is poorly understood. Our genetic screen identified the *Arabidopsis SPIKE1* (*SPK1*) gene whose loss-of-function mutations increased lateral root density and retarded gravitropic responses, as do *pin2* knockout mutations [3]. SPK1 belongs to the conserved DHR2-Dock family of Rho guanine nucleotide exchange factors [4–6]. The *spk1* mutations induced PIN2 internalization that was not suppressed by auxin, as did the loss-of-function mutations for Rho-like GTPase from Plants 6 (ROP6)-GTPase or its effector RIC1. Furthermore, SPK1 was required for auxin induction of ROP6 activation. Our results have established a Rho GTPase-based auxin signaling pathway that maintains PIN2 polar distribution to the plasma membrane via inhibition of its internalization in *Arabidopsis* roots. Our findings provide new insights into signaling mechanisms that underlie the regulation of the dynamic trafficking of PINs required for long-distance auxin transport and that link auxin signaling to PIN-mediated pattern formation and morphogenesis.

Results and Discussion

Genetic Screen for ROP6 Interactors Identified a Novel *spk1* Allele that Altered Root Development

The quintessential phytohormone auxin regulates a wide range of developmental and morphogenetic processes [7–11], which require polar auxin transport primarily determined by polar localization of the PIN-FORMED (PIN) auxin efflux carriers [12–14]. PIN polarization relies on asymmetric endocytosis and endosomal recycling of PIN proteins [1, 2, 15–19], yet the signaling mechanisms governing PIN trafficking and polarization remain poorly understood. In leaf pavement cells, auxin activates both the Rho-like GTPase from Plants 2 (ROP2)- and ROP6-GTPase pathways [20], and the ROP2 pathway mediates auxin inhibition of PIN1 endocytosis [20, 21]. ICR1 (interactor of constitutive active ROPs), a ROP effector, promotes endocytic recycling of PIN proteins in roots [22]. However, it is unknown whether ROP-based auxin signaling also regulates PIN distribution in a process that requires polar auxin transport within a plant tissue.

To identify new components of ROP signaling in *Arabidopsis*, we performed a genetic screen for mutations that enhanced cell shape changes induced by *ROP6* overexpression [23, 24] and characterized one of these mutations (see Figures S1A and S1B available online). The mutation also increased lateral root density and reduced the length of primary roots (Figures 1A–1C). Mapping and sequencing revealed that the mutation was a single base G-to-A substitution in the 28th exon of *SPIKE1* (*SPK1*) (Figure S1C). The mutation did not change the corresponding amino acid residue of 1,421 in the SPK1 protein (Figure S1C) but was predicted to cause aberrant messenger RNA splicing, generating two different truncated proteins (Figures S1D and S1E). We named the new allele as *spk1-4*. SPK1 belongs to the dock homology region 2 (DHR2)-type Dock family of Rho guanine nucleotide exchange factors (RhoGEFs) and acted as a GEF for ROPs in in vitro assays [6].

To confirm whether the root phenotype was caused by the *spk1-4* mutation, we obtained two null mutants, *spk1-1* and *spk1-5* (Figures S1C and S1F). Both *spk1-1* and *spk1-5* seedlings showed similar root phenotypes as *spk1-4* (Figures 1A–1C). The analysis of *SPK1p::GUS* suggests that SPK1 is expressed in roots and aerial organs (Figure S1J). Homozygous *spk1-4* showed normal aerial morphology as wild-type (WT) plants (Figures S1G–S1I). In contrast, homozygous *spk1-1* and *spk1-5* plants were extremely dwarfed (Figures S1G-S1I). Hence, we conclude that *spk1-4* is a partial loss-of-function mutation.

Mutations in *SPK1* Enhance PIN2 Internalization and Affect PIN2's Function in Roots

Although *spk1* null mutants have strong pleiotropic aerial phenotypes [5] (Figures S1G–S1I), the increased lateral root density resembles that of *pin2* knockout mutants in seedling stages or WT *Arabidopsis* seedlings treated with auxin [3, 25] (Figures S3E and S3F), which hinted to a possible involvement of SPK1 in the regulation of PIN2-mediated polar auxin transport. Consistent with a defect in polar auxin transport, *spk1-1* dramatically enhanced *DR5::GUS* expression along the steles of the primary roots and in the primordia of lateral roots in 6-day-old seedlings (Figures S2A–S2C). In roots, PIN2 is localized to the apical end of epidermal cells and the basal end of cortical cells in the root tips and thus participates in a connected circulatory auxin flow that is downward in the stele or cortex and upward in the epidermal cells [26, 27]. The *spk1* mutations reduced the amount of

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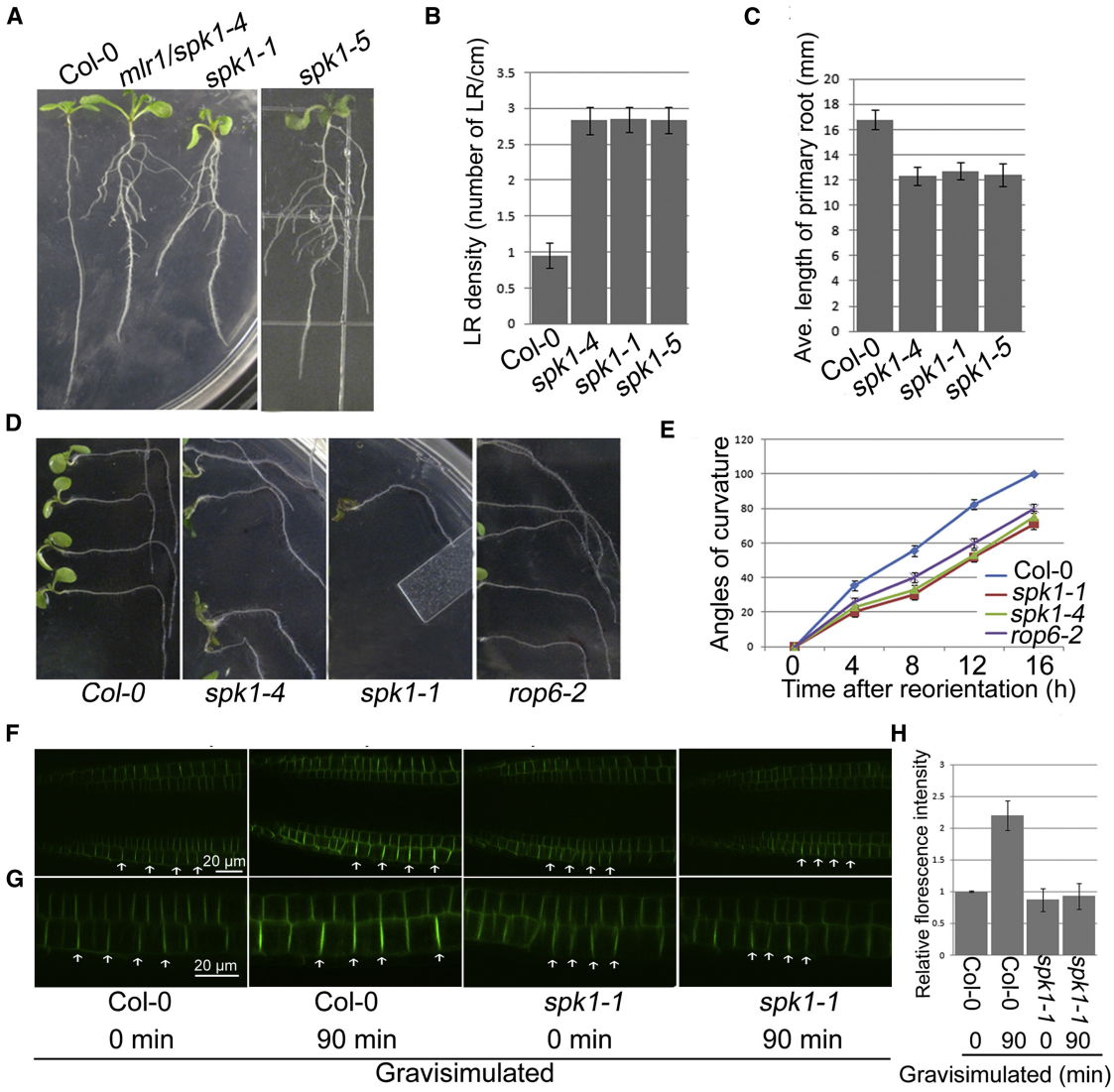

Figure 1. *spk1* Mutants Develop More Lateral Roots and Are Retarded in Gravitropic Responses

(A) Altered root development in 10-day-old seedlings of *spk1-1*, *spk1-4*, and *spk1-5* mutants.

(B) Quantification of lateral root density (LRs/cm primary root) in 10-day-old seedlings from Col-0, *spk1-1*, *spk1-4*, and *spk1-5*. Three *spk1* mutants had a similar lateral root density. *spk1* mutants showed more emerged lateral roots compared to WT ($p < 0.01$, t test). Error bars indicate SE; n = 20.

(C) Quantification of primary root length of 4-day-old seedlings from Col-0, *spk1-1*, *spk1-4*, and *spk1-5*. Three *spk1* mutants had a similar primary root length, which were significantly shorter than WT ($p < 0.01$, t test). Error bars indicate SE; n = 20.

(D) *spk1-4*, *spk1-1*, and *rop6-2* mutants showed less sensitivity to gravistimulation compared to WT. Four-day-old seedlings were rotated 90° for testing gravitropic response. The images were taken after 36 hr reorientation.

(E) Quantification of gravitropic response in Col-0, *spk1-4*, *spk1-1*, and *rop6-2*. Four-day-old seedlings were rotated 90°, and root tropic bending curvatures for Col-0, *spk1-4*, *spk1-1*, and *rop6-2* seedlings were measured at intervals of 4 hr. Statistical analysis indicated significant differences between Col-0 and all of the mutants ($p < 0.05$). Error bars represent SE (n = 60).

(F and G) PIN2 immunolocalization in WT and *spk1-1* seedlings. Four-day-old seedlings were gravistimulated for 0 or 90 min. After 90 min, PIN2 levels in the lower side of the root were more pronounced than those in the upper side in WT seedlings. However, in *spk1-1* mutant seedlings, PIN2 levels were not changed after 90 min gravistimulation.

(H) Quantification of relative florescence intensity of PIN2 levels in the lower-side epidermal cells after gravistimulation for 0 or 90 min in WT and *spk1-1* mutants ($p < 0.05$, t test).

See also Figure S1.

PIN2 polarly distributed to the plasma membrane (PM) in root epidermal cells, although the polarity of PIN2 localization was not affected (Figures 2A–2C). Furthermore, PIN2 was detected in intracellular compartments in *spk1-1* root cells, but not in WT cells (Figure 2A; Figure S3C). By inhibiting endosomal recycling, Brefeldin A (BFA) induces the accumulation of internalized PIN proteins in a compartment termed BFA bodies [1, 16]. PIN2 accumulation in BFA bodies increased in *spk1* mutants compared to WT (Figures 2D and 2E; Figure S3D). Following BFA washout, the PIN2-containing BFA bodies disappeared in almost 90% of both WT and *spk1-1* cells and no significant differences were found between WT

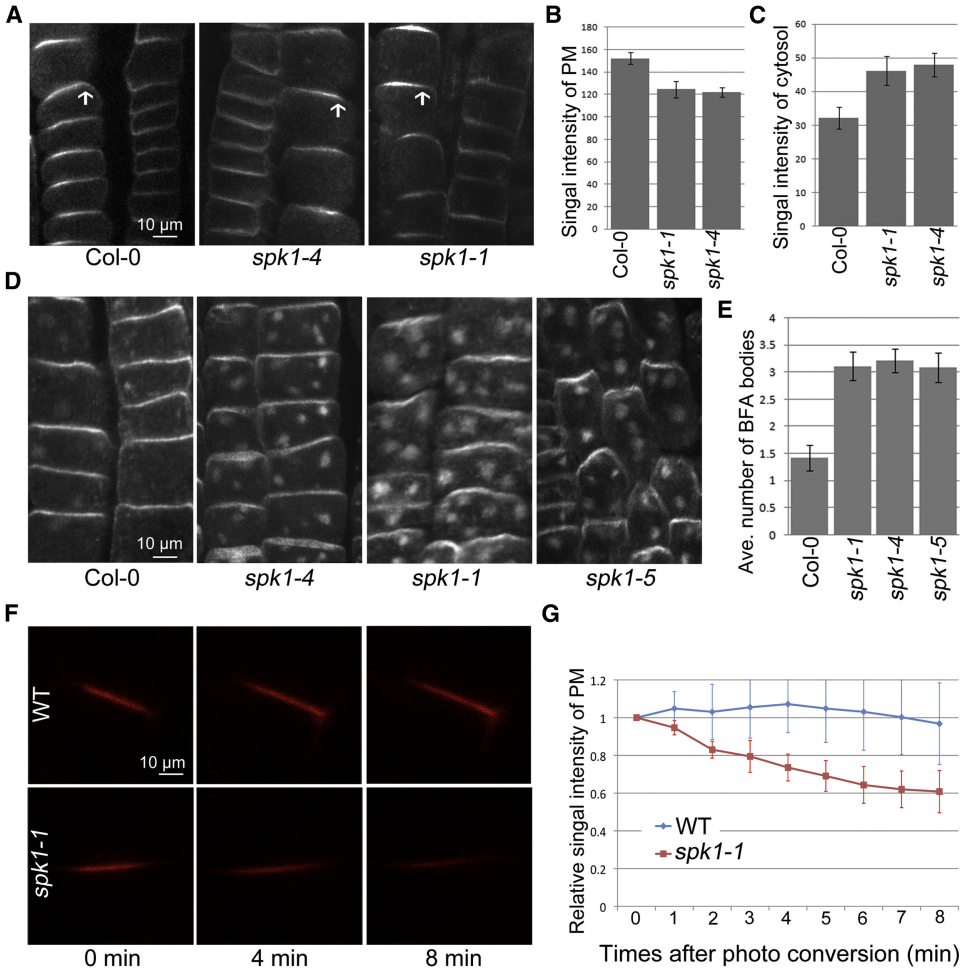

Figure 2. SPK1 Inhibits PIN2 Internalization in Root Epidermal Cells

(A) PIN2 subcellular distribution in WT and *spk1* mutants. PIN2 distribution to the apical PM side of root epidermal cells (see arrowhead) was reduced in *spk1* mutants compared to WT, whereas PIN2 distribution to the cytoplasm was increased in *spk1* cells.

(B) Quantification of PIN2 signal intensity at the PM of epidermal cells. Fluorescence was measured by calculating the mean fluorescent signal intensity on the PM of epidermal cells. The amount of PIN2 at the PM was significantly reduced in cells of *spk1* mutants compared to WT ($p < 0.01$, t test). At least 100 cells from three independent experiments were analyzed for each assay. Error bars represent SD.

(C) Quantification of PIN2 signal intensity in the cytoplasm of epidermal cells. The amount of PIN2 in the cytoplasm was significantly increased in cells of *spk1* mutants compared to WT ($p < 0.01$, t test). At least 100 cells from three independent experiments were analyzed for each assay. Error bars represent SD.

(D) Internalization of PIN2 into BFA bodies was increased in epidermal cells of all three *spk1* mutants compared to WT.

(E) Quantification of BFA compartments in root epidermal cells of WT and *spk1* mutant. *spk1* mutations increased PIN2-containing BFA bodies compared to WT ($p < 0.01$, t test). At least 150 cells from three independent experiments were analyzed for each assay. Error bars represent SD.

(F) A time-course analysis of PIN2-EosFP internalization from the PM after UV-induced green-to red photoconversion. Decrease in the photoconverted fluorescent signal at the PM was accelerated in *spk1-1* cells.

(G) Quantification of PM signal shown in (F). Relative signal intensity was calculated by absolute values of intensity divided by the value of intensity in time = 0 after photoconversion ($p < 0.01$, t test). At least 50 cells from three independent experiments were analyzed for each assay. Error bars represent SD.

See also Figure S2.

and *spk1-1* (Figures S2F and S2G). These results imply that SPK1 inhibits PIN2 internalization but is not required for PIN2 recycling. We also tracked the internalization of photoconverted PIN2-EosFP that was initially localized to the PM [18]. The photoconverted PM PIN2-EosFP signal decreased much more rapidly in *spk1-1* cells than in WT cells (Figures 2F and 2G). Moreover, uptake of the endocytic tracer FM4-64 was clearly increased in roots of both *spk1-1* and *spk1-5* mutants compared to WT (Figures S2D and S2E). Furthermore, the *pin2-1 spk1-1* double mutant showed a root phenotype identical to that of the *spk1-1* or *pin2-1* single mutant (Figures S3E and S3F) [3]. Taken together, our results suggest that SPK1 inhibits the internalization of PIN2 in root epidermal cells, consequently promoting PIN2 retention at the PM.

PIN2 is also required for gravitropic curvature of *Arabidopsis* roots by regulating auxin redistribution, and dynamic changes in PIN2 protein redistribution are important for its function in root gravitropism [26–29]. The *spk1-1* and *spk1-4* mutant seedlings exhibited significant decreases in gravitropic bending at all time points after seedling reorientation (Figures 1D and 1E). *DR5::GFP* is expressed symmetrically in the root columella in vertically growing seedlings, with limited expression in the epidermis beyond the root tip (Figure S3G). Within 4 hr after horizontal reorientation of

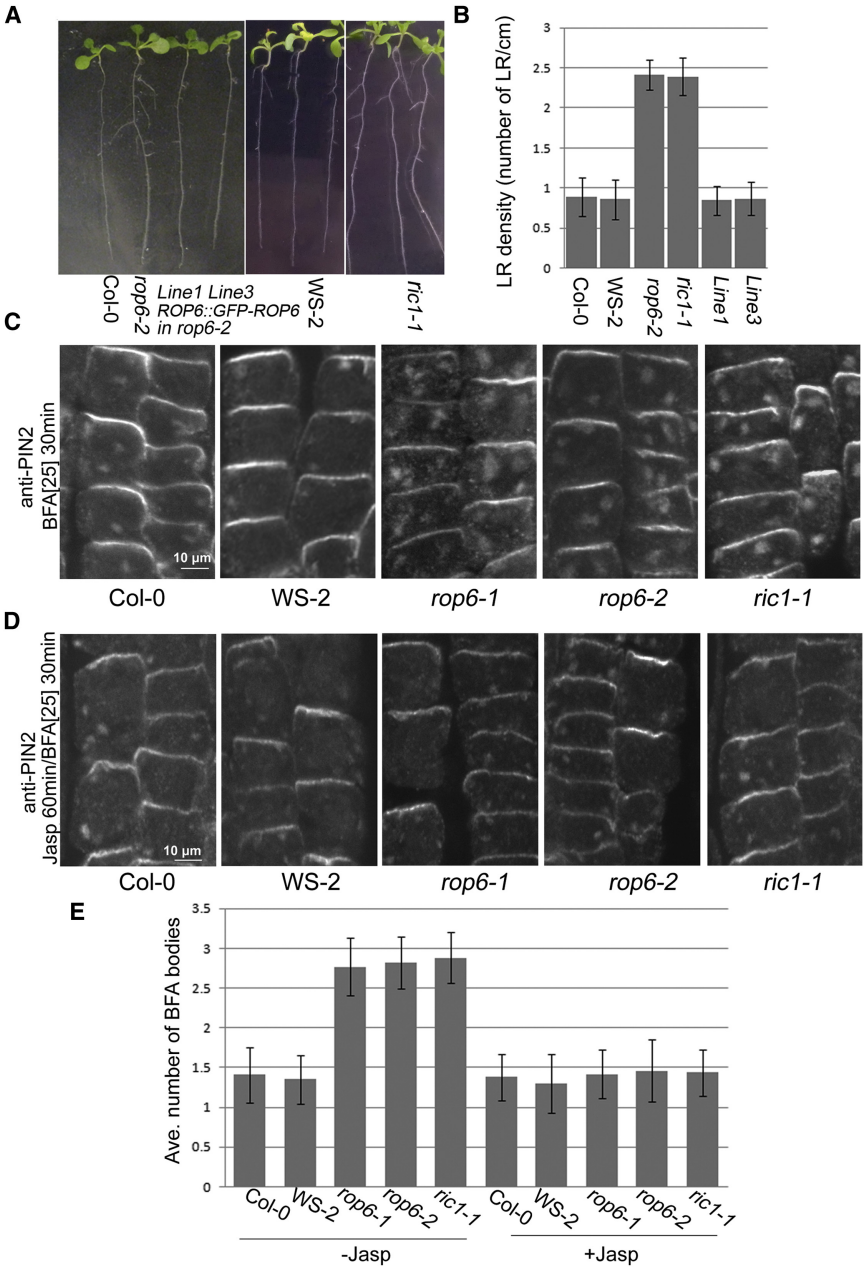

Figure 3. ROP6/RIC1 Inhibits PIN2 Internalization through Stabilization of F-actin

(A) Loss-of-function mutations for ROP6 and RIC1 increased lateral root formation in 10-day-old seedlings. Root phenotypes were observed in 10-day-old seedlings from Col-0, *rop6-2*, *rop6-2* lines complemented with *pROP6::GFP-ROP6*, WS-2, and *ric1-1* (WS-2 background). The *rop6-2* mutant (Col-0 background) was complemented by transforming the *pROP6::GFP-ROP6* construct into *rop6-2* plants. Eight out of 11 independent transformed lines showed recovery of the lateral root phenotype. Two of these lines (#1 and 3) were chosen for phenotype analysis.

(B) Quantification of lateral root density (LRs/cm primary root) in 10-day-old seedlings from Col-0, WS-2, *rop6-2*, complemented *rop6-2* line and *ric1-1. rop6-2* and *ric1-1* mutants showed more lateral roots compared to WT, respectively ($p < 0.01$, t test). Error bars indicate SE; n = 20.

(C) Increased BFA-induced internalization of PIN2 in *rop6-1*, *rop6-2*, and *ric1-1* mutants compared to WT.

(D) Jasplakinolide (500 nM) treatment could reduce BFA-induced internalization of PIN2 in *rop6-1*, *rop6-2*, and *ric1-1* mutants.

(E) Quantification of BFA bodies in WT, *rop6-1*, *rop6-2*, and *ric1-1* mutant lines before or after Jasplakinolide (500 nM) treatment. All mutants had more PIN2-containing BFA bodies within 30 min before Jasplakinolide treatment compared to WT ($p < 0.01$, t test). Jasplakinolide treatment suppressed the increase in PIN2 internalization into BFA bodies in *rop6-1* (WS-2 background), *rop6-2*, and *ric1-1* cells. At least 150 cells from three independent experiments were analyzed for each assay. Error bars represent SD.

See also Figure S3.

control seedlings, *DR5::GFP* exhibited preferential expression along the lower side of the root cap and epidermal tissues that extends 150 to 200 μm from the root tip into the root distal elongation zone, where asymmetric growth drives gravitropic bending (Figure S3H) [1]. In contrast, the asymmetric *DR5::GFP* expression at the lower side of the lateral root cap in *spk1-1* seedlings were less pronounced, with expression extending at a shorter distance than in control seedlings (Figure S3H). Within 90 min after gravistimulation, PIN2 signals were higher at the lower side than at the upper side of horizontally positioned roots, and this difference was most pronounced in epidermal cells [30] (Figures 1F–1H). In contrast, PIN2 signals in *spk1-1* seedlings showed similar levels both at the lower side and the upper side (Figures 1F–1H). These results suggest that SPK1 is required for PIN2-mediated root gravitropic responses.

ROP6 Regulates PIN2 Dynamic Distribution

We reasoned that as a RopGEF SPK1 would activate a ROP GTPase to modulate auxin transport in roots. From available *Arabidopsis* T-DNA insertional *rop* mutants, we found that indeed a ROP6 null mutant *rop6-2* exhibited a phenotype similar to that of *spk1-1* (Figures 3A and 3B; Figures S3A and S3B). In *rop6-2* seedlings, lateral root density increased by about 130% as compared to WT (Figure 3B). *ROP6p::GFP-ROP6* complemented the *rop6-2* root phenotype, confirming that ROP6 regulates lateral root density (Figures 3A and 3B). Furthermore, *rop6-2* seedlings were retarded in root gravitropic responses, as do *spk1* mutant seedlings (Figures 1D and 1E).

Consistent with the root phenotype, the accumulation of PIN2 protein in BFA bodies was greatly increased in both *rop6-1* and *rop6-2* root cells compared to WT (Figure 3C and 3E). In contrast, *ROP6* overexpression reduced the uptake of the endocytic tracer FM4-64 and PIN2 internalization into BFA bodies in root cells (Figures S3I, S3L, and S4A). The analysis of *ROP6p::GFP-ROP6* lines suggested that *ROP6* is preferentially expressed in lateral root primordia and root cortical and epidermal cells where *PIN2* is expressed (Figures S4B and S4C). Coimmunostaining revealed

that ROP6 colocalized with PIN2 at the apical side of root epidermal cells (Figure S4D). Taken together, these results show that ROP6 regulates PIN2 subcellular distribution and consequently the function of PIN2 in the distribution of auxin in roots.

ROP6 Regulates PIN2 Distribution Likely by Promoting the Stabilization of Actin Microfilaments

In pavement cells, ROP6 promotes microtubule organization [24], but treatment of *ROP6*-overexpressing root cells with microtubule (MT)-destabilizing drug oryzalin did not change PIN2 internalization (Figures S3I, S3J, and S3L), suggesting that ROP6 inhibits PIN2 internalization in a MT-independent manner. The ROP2-RIC4 pathway promotes PIN1 polarization to the PM via the stabilization of cortical actin microfilaments in pavement cells [21]. Interestingly, treatments with 100 nM latrunculin B induced PIN2 accumulation in BFA bodies in *ROP6*-overexpressing root cortical cells (Figures S3I, S3K, and S3L), whereas Jasplakinolide (Jasp), which stabilizes F-actin, suppressed the increase in PIN2 accumulation in BFA bodies in *rop6-2* cells (Figures 3C–3E).

We next asked whether ROP6 regulates actin stabilization through its effector RIC1 [24]. *ric1-1* mutants showed an increase in lateral root density compared to WT (Figures 3A and 3B). PIN2 aggregation into BFA bodies was also greatly enhanced in *ric1-1* root cells (Figures 3C and 3E). *RIC1* overexpression inhibited the uptake of the endocytic tracer FM4-64 and PIN2 aggregation into BFA bodies, as did *ROP6* overexpression (Figures S3I, S3L, and S4A). Moreover, treatments with latrunculin B but not oryzalin restored BFA-induced aggregation of PIN2 in *RIC1*-overexpressing root cells (Figures S3I to S3L). Treatments with Jasp suppressed the increase in PIN2 aggregation into BFA bodies in *ric1-1* cells (Figures 3C–3E). Taken together, our results suggest that the ROP6-RIC1 pathway inhibits PIN2 internalization through the stabilization of actin filaments in roots.

SPK1 Interacts with the Inactive Form of ROP6 and Is Required for ROP6 Activation In Vivo

We tested the hypothesis that SPK1 is the direct activator of ROP6 in the regulation of PIN2 distribution. First, in vitro pull-down assays showed that the DHR2 GEF catalytic domain of SPK1 preferentially interacted with the GDP- but not GTP-bound form of ROP6 (Figure 4A), which is expected if SPK1 acts as a GEF for ROP6. Second, the preferential SPK1 interaction with the inactive form of ROP6 was confirmed using an in vivo fluorescence resonance energy transfer (FRET) analysis (Figures S4E and S4F). Third, coimmunoprecipitation (coIP) assay further confirmed that SPK1 interacted with ROP6 in vivo (Figure 4B). Finally, the *spk1-1* mutation greatly reduced the amount of active ROP6 in vivo, suggesting that SPK1 is required for ROP6 activation in vivo (Figure 4C).

The SPK1-ROP6 Pathway Is Required for Auxin-Mediated Inhibition of PIN2 Internalization

In pavement cells, auxin activates the ROP6-RIC1 pathway [20]. We also found that auxin treatments increased the amount of active ROP6 in *GFP-ROP6* plants (Figure 4C) and that auxin-dependent increase in ROP6 activity was largely abolished in the *spk1-1* mutant (Figure 4C). Consistent with previous report [1], naphthalene acetic acid (NAA) inhibited BFA-induced PIN2 aggregation in WT roots (Figures 4D and 4E). However, in *spk1-1*, *rop6-1*, and *ric1-1* root cells, NAA was ineffective in inhibiting BFA-induced aggregation of PIN2 (Figures 4D and 4E). These results indicate that the SPK1-ROP6 pathway is required for auxin-mediated inhibition of PIN2 internalization and provide strong support for our hypothesis that auxin inhibits PIN2 internalization by activating the SPK1-ROP6-RIC1 pathway.

Conclusions

Our findings here have established a ROP GTPase-based auxin signaling pathway regulating the dynamic subcellular distribution of PIN2 that is critical for polar auxin transport in roots (Figure 4F). The SPK1-ROP6-RIC1 pathway inhibits PIN2 internalization, which modulates the auxin distribution that affects root growth and lateral root formation and the auxin redistribution during gravitropic responses. This work, together with recent complementary studies on ROP regulation of PIN1 and PIN2 distribution in leaf and root cells [21, 31], suggest that the ROP-based auxin signaling that regulates PIN internalization is a widespread mechanism that modulates polar auxin transport in plants.

Our data imply that ROP6/RIC1-dependent auxin signaling pathway inhibits PIN2 internalization by stabilizing actin filaments in root cells. In leaf pavement cells, the ROP2-RIC4 pathway has been shown to induce F-actin accumulation, leading to the inhibition of PIN1 internalization required for PIN1 polarization in the lobe tips [21]. These findings are consistent with several studies suggesting that the stabilization of F-actin inhibits PIN endocytosis [21, 32, 33]. It will be important to determine whether cell polarity signaling that targets actin polymerization and dynamics is a common design principle for the regulation of endocytosis in eukaryotic systems.

Revolving around the emerging general theme of ROP-based regulation of PIN distribution in plants, the detailed mechanisms for this regulation appear to differ within cell types and tissues. In leaf PCs, ABP1 is required for the activation of the ROP2-RIC4 pathway that inhibits PIN1 endocytosis [20, 21], whereas in roots it is the ROP6-RIC1 pathway that inhibits PIN2 internalization and is negatively regulated by ABP1 [31]. The tissue-specific detailed mechanisms may reflect the different auxin concentrations required for the activation of these pathways in different tissues. In PCs, ROPs respond to very low auxin concentrations [20]; however, higher auxin concentrations are required for inhibition of endocytosis in roots [1] (Figures 4D and 4E). In roots, ROP6 only inhibits PIN internalization and has no effects on PIN recycling or polarity, whereas another ROP effector ICR1 is required for PIN1 recycling and polarization but does not regulate its endocytosis [22]. In PCs, ROP2 is required for PIN1 polarization [20, 21] and may also regulate its recycling. Future studies should determine whether ROP-based auxin signaling provides a universal mechanism for polar distribution of PIN and polar auxin transport and unveil the reasons behind the tissue or cell-specific mechanisms for the regulation of PINs.

Supplemental Information

Supplemental Information includes four figures, one table, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2012.05.019.

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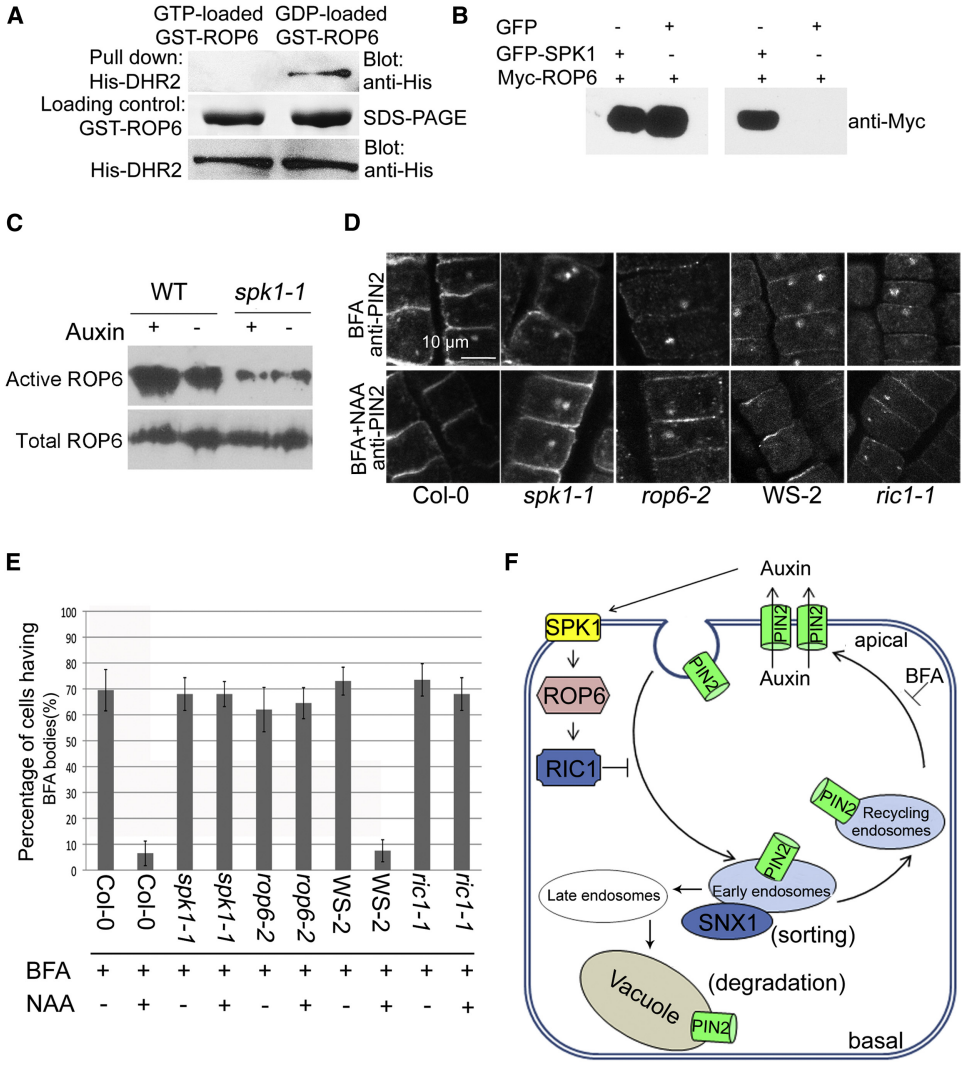

Figure 4. SPK1 Interacts with ROP6 and Is Required for Auxin-Mediated Activation of ROP6 and Inhibition of PIN2 Internalization

(A) Recombinant DHR2 domain of SPK1 interacted with GDP-loaded ROP6. GTP-bound or GDP-bound GST-ROP6 was used in GST pull-down assays with recombinant His_6-DHR2. Only GDP-bound GST-ROP6 interacted with His_6-DHR2.

(B) SPK1-ROP6 interaction was analyzed using coIP assay in *N. benthamiana*. The GFP or Myc-ROP6 and GFP-SPK1or Myc-ROP6 constructs were coexpressed in *N. benthamiana* by the infiltration method, respectively, and total protein extracts were subjected to immunoprecipitation using anti-GFP antibody conjugated with protein A agarose beads. The resultant immunocomplexes were analyzed on western blot using anti-Myc antibody.

(C) *spk1-1* mutation reduced ROP6 activity and suppressed auxin-induced activation of ROP6. Protoplasts from 10-day-old seedlings of transgenic GFP-ROP6 or *spk1*-1/GFP-ROP6 were treated with 100 nM NAA for 10 min. GTP-bound active GFP-ROP6 and total GFP-ROP6 (GDP and GTP forms) were analyzed as described in the text. Three independent experiments produced similar results, and results from one representative experiment are shown.

(D) Effects of auxin on PIN2 internalization in root epidermal cells of WT (Col-0 and WS-2), *spk1-1*, *rop6-2*, and *ric1-1*. Auxin (NAA) inhibited the accumulation of PIN2 in BFA bodies in WT cells. However, auxin did not inhibit PIN2 accumulation in these structures in *spk1-1*, *rop6-2*, and *ric1-1* mutants.

(E) Quantitative data showing percentages of cells containing BFA bodies. Error bars represent SE.

(F) A model for the auxin-mediated maintenance of polar PIN2 distribution via a ROP GTPase-based auxin signaling pathway that inhibits PIN internalization. Our data demonstrate that auxin-mediated inhibition of PIN2 internalization is controlled by the SPK1-ROP6-RIC1 signaling pathway. We propose that auxin activates the SPK1-ROP6-RIC1 pathway and leads to the subsequent inhibition of PIN2 internalization. This ROP6 signaling-based localized inhibition of PIN2 internalization retains PIN2 in the PM, which provides a positive feedback mechanism for the maintenance of the polar PIN2 distribution to the PM. See also Figure S4.

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