# Gravity-induced PIN transcytosis for polarization of auxin fluxes in gravity-sensing root cells

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**Auxin is an essential plant-specific regulator of patterning processes that also controls directional growth of roots and shoots. In response to gravity stimulation, the PIN3 auxin transporter polarizes to the bottom side of gravity-sensing root cells, presumably redirecting the auxin flux toward the lower side of the root and triggering gravitropic bending. By combining live-cell imaging techniques with pharmacological and genetic approaches, we demonstrate that PIN3 polarization does not require secretion of de novo synthesized proteins or protein degradation, but instead involves rapid, transient stimulation of PIN endocytosis, presumably via a clathrin-dependent pathway. Moreover, gravity-induced PIN3 polarization requires the activity of the guanine nucleotide exchange factors for ARF GTPases (ARF-GEF) GNOM-dependent polar-targeting pathways and might involve endosome-based PIN3 translocation from one cell side to another. Our data suggest that gravity perception acts at several instances of PIN3 trafficking, ultimately leading to the polarization of PIN3, which presumably aligns auxin fluxes with gravity vector and mediates downstream root gravitropic response.**

polarity | exocytosis | gravitropism | plant cell biology

Plants have evolved a profound phenotypic plasticity, enabling growth to adapt to changes in environmental conditions. The complex developmental reprogramming often involves resetting of developmental fate and polarity of cells within differentiated tissues. The local biosynthesis of the phytohormone auxin and its directional intercellular transport are essential in these processes because they provide positional information and link cell polarity with tissue patterning (1–3). PIN proteins are cellular auxin export carriers and their polar subcellular localization at the plasma membrane determines the direction of the intercellular auxin flow (4, 5).

PIN proteins undergo constitutive endocytic recycling between the plasma membrane and the endosomal compartments (6, 7), but the functional role of this recycling mechanism is still unclear. A plausible assumption is that constitutive trafficking regulates the cellular auxin transport rates (6) and accounts for the flexibility needed for the rapid PIN polarity changes that allow the auxin flow to be quickly redirected in response to various signals, including environmental or developmental cues (8–10). Indeed, dynamic PIN translocation between different cell sides (termed transcytosis) can be pharmacologically induced in plant cells and might account for rapid PIN alterations during plant development (9, 10). Rapid changes in PIN polarities occur during embryonic development (11), aerial and underground organogenesis (12–15), vascular tissue formation (16), and root gravity responses (17, 18). However, the underlying mechanism of these PIN polarity alterations still needs to be demonstrated. The developmentally regulated changes in PIN polarity presumably redirect auxin fluxes and, subsequently, trigger alterations in the developmental programs (19–21).

An intriguing example of PIN polarity reorganization relates to the perception and response to environmental stimuli. For example, when the root is reoriented in a horizontal position, gravity-sensing statoliths in the columella cells sediment toward the new bottom side of these cells and PIN3 relocalizes from its originally uniform distribution to this side (17, 18, 22). The asymmetric repositioning of PIN3 is presumably followed by a downward auxin flow, leading to auxin accumulation at the bottom side of the root and, consequently, to asymmetric tissue growth and downward root bending (23). Despite the fundamental importance of PIN-dependent auxin transport for plant gravitropic responses, the underlying mechanism for PIN3 polarization remains to be established.

Here, we examined the cellular and molecular mechanism for PIN3 polarization in response to gravity. Our data suggest that gravity induces PIN3 internalization and its alternative recruitment to an ARF-GEF (guanine nucleotide exchange factors for ARF GTPases) polar-targeting machinery. Moreover, live imaging reveals endosome-based translocation of PIN3 proteins between different cell sides. Our data suggest that gravity modulates multiple steps of PIN3 trafficking leading to PIN3 transcytosis that has presumably physiological importance for the redirection of auxin fluxes during gravitropic response.

## Results and Discussion

**Gravity-Sensing Columella Cells Adjust Plant Growth by Redundant Pathways for Auxin Distribution.** Despite the anticipated importance of PIN3 for root gravitropism and its demonstrated role in shoot gravitropism, *pin3* mutant roots are only marginally defective in responding to gravity (17) (Fig. 1 *A* and *B*); pharmacological inhibition of auxin transport strongly interferes with root gravitropism (24), suggesting that functional redundancy among PIN family members (25, 26) might mask the importance of PIN3 polarization in gravity-sensing columella cells. In accordance with this assumption, we found that PIN3 and its closest homolog, PIN7, display partially overlapping expression patterns in columella cells. Expression of the functional PIN3-GFP (27) under its endogenous promoter was strong in the two layers of columella cells below the columella initials (in C1 and C2) (Fig. 1*C*). The expression domain of PIN7-GFP (25) under its own promoter was shifted one cell file below that of PIN3 (C2 and C3) (Fig. 1*E* and Fig. S1*A*). Notably, PIN7-GFP expanded into the PIN3 expression domain in *pin3* mutants (Fig. S1*B*), indicating a PIN7-dependent compensation of auxin flux in *pin3* mutant columella cells. Consistently, the gravitropic response defects of the *pin3 pin7* double-mutant seedlings were stronger than those of either single mutant (Fig. 1 *A* and *B*). Other PIN proteins, such as PIN4, which are also produced in the root tip (28), might contribute to gravity-induced auxin redistribution. However, higher order *pin* mutants, such as *pin3 pin4 pin7*, display strong developmental defects (25, 26);

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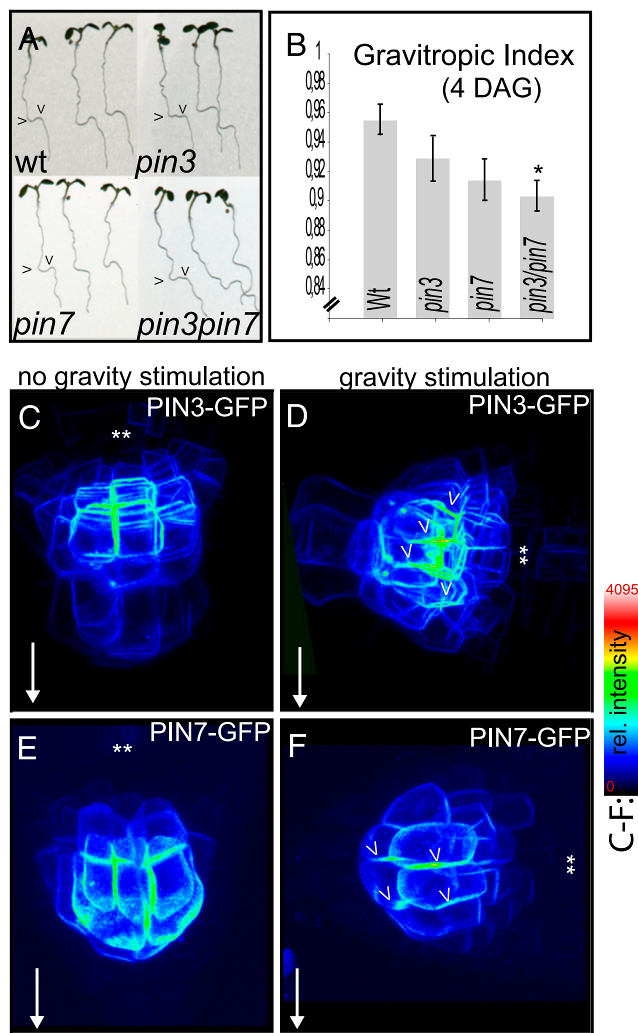


**Fig. 1.** Redundant pathways for directional auxin efflux in gravity-sensing columella cells. (*A* and *B*) The gravitropic response of *pin3 pin7* double mutants is reduced compared with wild-type, *pin3* or *pin7* single mutants (*A*). Plates were turned twice by 90° (indicated by arrowheads). The gravitropic response was significantly (error bars represent SD; $^*P < 0.05$) affected in *pin3 pin7* double mutants as early as 4 d after germination (*B*). (*C–F*) Functional PIN3-GFP (*C* and *D*) and PIN7-GFP (*E* and *F*) transgenic seedlings without (*C* and *E*) and with a gravity stimulus for 30 min (*D* and *F*). Arrowheads mark the preferential polarization of PIN3-GFP and PIN7-GFP. A semiquantitive color-coded heat-map of *z*-stack maximum projections (10 sections/each 2 μm) is provided (*C–F*). Arrows and asterisks indicate the gravity vector and the position of the quiescent center, respectively.

hence, their contribution to the gravitropic response is difficult to assess. Nevertheless, our findings show that PIN auxin transporters act redundantly in gravity-sensing columella cells during the gravity response.

Endogenous PIN3 localizes nonpolarly in columella cells, but acquires a polarized distribution, in alignment with the gravity vector, after reorientation of the root in the gravitational field (17). We established a semiquantitative, confocal microscope-based, live-cell imaging technique to detect gravity-induced signal enrichment of PIN3-GFP at the bottom side of cells (Fig. 1*D*). Visualization of fluorescent intensity using a color-coded heat-map and maximum projections of several optical sections enabled us to observe that, contrary to previous descriptions, in nonstimulated columella cells PIN3-GFP is not always symmetrically localized, but instead, occasionally shows asymmetric localization patterns (Fig. S1*C*). This transient PIN3 polarization in nonstimulated roots could generate asymmetric auxin fluxes toward the root-elongation zone that might induce wavy growth in wild-type seedlings, which appears to be developmentally important for soil penetration and alternative spacing of lateral roots (29). Alternatively, mechanical forces during the growth on the solid media might also stimulate growth responses involving transient PIN3 polarization. Although the polarization of PIN3 was observed in ≈10% of nonstimulated seedlings, it was much stronger in ≈40% of the seedlings within 30 min of gravity stimulation (Fig. S1*C*). Furthermore, the direction of the polarization was random in nonstimulated seedlings, whereas PIN3 was clearly aligned in the direction of gravity in gravity-stimulated roots (Fig. S1*C*). It is very likely that our semiquantitive imaging approach is not able to detect partial PIN3 polarization events that might have already significant impact on asymmetric auxin redistribution. Nevertheless, our approach reveals that the majority of the root columella cells display pronounced gravity-induced PIN3 polarization earlier then 30 min after stimulation.

To investigate the potential redundancy of PIN3 and PIN7 in wild-type seedlings, we also analyzed fluorescent enrichment of PIN7-GFP in the presence and absence of gravity stimuli. Gravity stimulation not only polarized PIN3-GFP (Fig. 1 *C* and *D*), but also induced a similar asymmetry in the PIN7-GFP distribution in root columella cells (Fig. 1 *E* and *F*). Our data suggest that gravity induces polarization of both PIN3 and PIN7 in columella cells. Taking these data together, we assume that PIN3 and PIN7 act in a functionally redundant manner for the redistribution of auxin for the root gravitropic response.

**PIN3 Polarization Is Independent of de Novo PIN3 Secretion.** To investigate the cellular mechanisms that might mediate PIN polarization in gravity-sensing columella cells, we initially tested whether the secretion of de novo synthesized PIN3-GFP is required for PIN polarization in response to gravity. When the PIN3-GFP signal was broadly photobleached, no significant PIN3-GFP secretion was detected within the 30-min time-frame after gravity stimulation, during which PIN3-GFP polarization is observed (Fig. S2 *A*, *B*, and *F*). Moreover, cycloheximide (CHX), an inhibitor of protein-biosynthesis, did not significantly interfere with the polar deposition of PIN3-GFP (Fig. S2*C*). To further analyze whether initial PIN3 polarization is indeed independent of PIN3 expression after gravity stimuli, we constructed a photoconvertable PIN3-EosFP. The green-to-red photoconversion of PIN3-EosFP enabled us to follow the activated PIN3-EosFP (red) independently of de novo secretion. Nonstimulated seedlings showed no or only minor and random polarization of photoconverted PIN3-EosFP ($n = 8$) (Fig. 2 *C* and *D* and Fig. S2*E*), but in gravity-stimulated *PIN3-EosFP*–expressing seedlings, the photoconverted PIN3-EosFP polarized clearly in more than 50% of the analyzed seedlings ($n = 12$) (Fig. 2 *A* and *B* and Fig. S2*D*). Collectively, our data indicate that de novo secretion of the PIN3 protein is not required for the initial polarization in response to gravity.

**Gravity Does Not Induce Enhanced PIN3 Degradation.** The vacuolar targeting of PIN2 has been shown to be enhanced in root epidermal cells during the gravity response, presumably maintaining the asymmetric auxin distribution (30, 8). Therefore, we examined the feasible scenario that differential PIN3 internalization at certain cell sides and subsequent lytic degradation might account for PIN3 polarization in response to gravity (17). We used an established vacuolar accumulation assay (8) to investigate the PIN protein degradation in gravity-sensing columella cells. PIN3-GFP occurred only very faintly in vacuoles after 3 h, whereas PIN7-GFP had a substantial turnover in the same time frame (Fig. 2 *E* and *G*). Importantly, gravity stimulation did not affect the vacuolar accumulation of PIN3-GFP or PIN7-GFP (Fig. 2 *F* and *H*).

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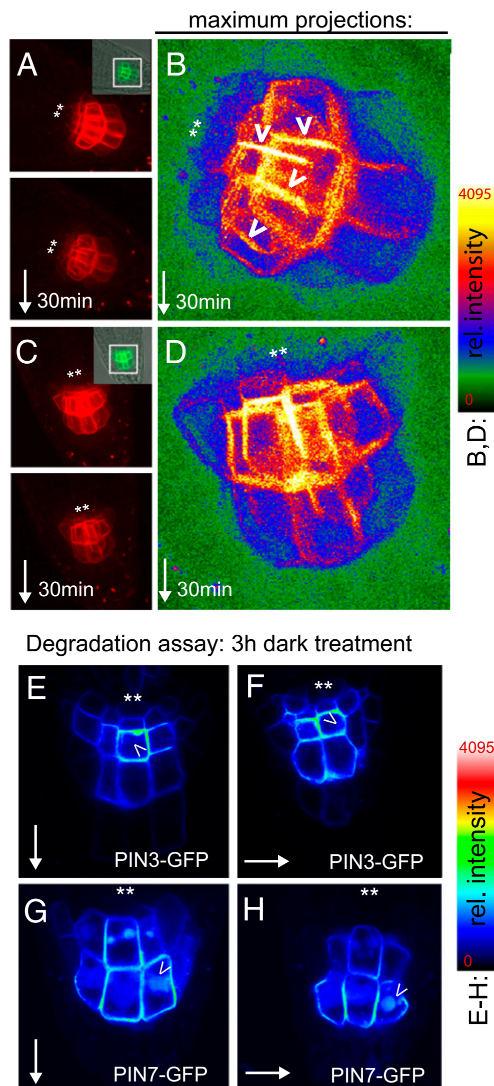


Fig. 2. PIN polarization requires recycling. (*A–D*) Photoconvertible *PIN3:PIN3-EosFP*–expressing seedlings. PIN3-EosFP (green to red) was photoconverted to track PIN3 proteins independently of secretion (*A* and *C*; *Inset* shows PIN3-EosFP before conversion in green) and subsequently the seedlings were gravity-stimulated by 90° (*B*). Control seedlings were not stimulated after the photoconversion (*D*). Maximum projections (*z*-stack of 10 optical sections; each 2 μm) in conjunction with semiquantitive imaging techniques were used to evaluate the preferential PIN3-EosFP polarization after 30 min of gravity stimulation. (*E–H*) Vacuolar accumulation of PIN3-GFP (*E* and *F*) and PIN7-GFP (*G* and *H*) without (*E* and *G*) and in the presence of a gravity stimulus (*F* and *H*) was addressed with a dark treatment for 3 h. Arrows and asterisks indicate the gravity vector and the position of the quiescent center, respectively. Arrowheads highlight preferential PIN3 polarization (*B*) or vacuolar accumulation (*E–H*).

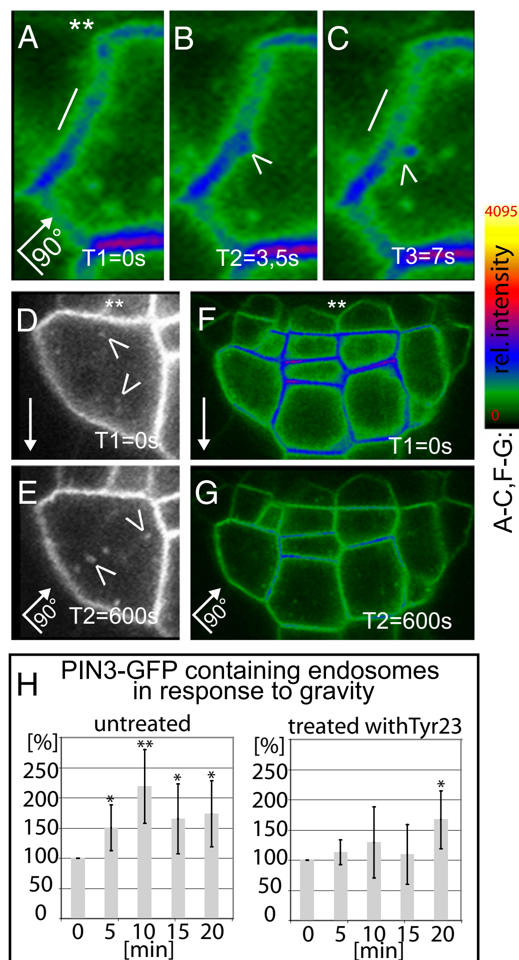


Fig. 3. Gravity-induced PIN3 internalization. (*A–C*) Loss of PIN3-GFP intensity at the plasma membrane was correlated with endosomal proximity. Seedlings were gravity stimulated and frames were taken every 3.5 s. White bars highlight a region of PIN3 internalization and arrowheads mark endosomal proximity. (*D* and *E*) Nonstimulated seedlings show weak endosomal PIN3-GFP signal (*D*). Upon gravity stimulation (600 s), the endosomal PIN3 signal was enhanced (*E*). (*F* and *G*) Individual seedlings were gravity stimulated for 90°. PIN3 signal intensity at the plasma membrane (*F*) decreased after 600 s of gravity stimulation (*G*). (*H*) PIN3-GFP containing endosomes were counted 0, 5, 10, 15, and 20 min after gravity stimulation ($n > 30$ cells). Tyrphostin A23 treatment partially suppressed gravity-induced PIN3 internalization into endosomes (error bars represent SD; $*P < 0.05$; $**P < 0.001$). Arrows mark the gravity vector and asterisks the position of the quiescent center.

These findings suggest that, although the lytic degradation rates of PIN3-GFP and PIN7-GFP might differ, neither is visibly influenced by gravity stimulation.

**Gravity Induces PIN3 Internalization.** The observations that PIN3 protein polarization in response to gravity requires neither secretion of de novo synthesized proteins nor increased lytic degradation hint at modulation of the trafficking of the preexisting pool of PIN proteins as the most likely mechanism for PIN polarization. Therefore, we investigated early trafficking events of PIN3-GFP in response to gravity by using live-cell imaging of PIN3-GFP during constant 90° gravity stimulation. After 5 to 10 min of gravity stimulation, an enhanced internalization of PIN3-GFP was observed from the plasma membrane into the endosomal compartments (Fig. 3 *A–C*), leading to a more pronounced occurrence of PIN3-GFP in endosomes (Fig. 3 *D*, *E*, and *H*). A decrease in signal intensity at the plasma membrane after 10 to 20 min was detected in 90° gravity-stimulated seedlings (Fig. 3 *F* and *G*). To control the potential effects of mechanical stimulations during constant microscopical survey, we also examined seedlings grown on agar plates that were either turned by 90° or 360° (negative control) and allowed to grow for 15 min before confocal microscope-based observation. In seedlings turned for 360°, no visible alterations in the PIN3-GFP localization were observed, but in most 90° gravity-stimulated seedlings, the PIN3-GFP endosomal signals increased and the average PIN3-GFP signal at the plasma membrane decreased in ≈20% of the seedlings

($n$ = 20; data not shown). Overall, these observations show that gravity perception induces a substantial internalization of PIN3 from the plasma membrane into the endosomal compartments.

The internalization of the auxin efflux carriers PIN1 and PIN2 has been shown to require the activity of clathrin at the plasma membrane. Moreover, PIN2 internalization in root epidermal cells can be inhibited by tyrphostin A23, an inhibitor of clathrin-dependent processes, but not by tyrphostin A51 that is structurally related to tyrphostin A23, but fails to bind to clathrin adaptors (7). Gravity-induced internalization of PIN3-GFP was suppressed by tyrphostin A23 treatments (Fig. 3*H*), indicating that clathrin function is required for the rapid internalization of PIN3 following a gravity stimulus. Furthermore, the polarization of the endogenous PIN3 protein, the asymmetric auxin distribution, and gravitropic bending were inhibited by tyrphostin A23, but not tyrphostin A51 (Fig. S3 *A–D*). In conclusion, these experiments illustrate that tyrphostin A23 inhibits asymmetric auxin distribution and gravitropic response, presumably by inhibiting various clathrin-mediated processes, including PIN3 internalization. This finding indicates not only that gravity induces PIN3 internalization but that PIN3 internalization might be required for gravity-induced PIN3 polarization.

**PIN3 Polarization Requires Brefeldin A-Sensitive Endocytic Recycling.** Our results suggest that gravity-induced, clathrin-mediated PIN3 internalization, but not degradation, is needed for PIN3 polarization. Thus, PIN3 internalization per se might probably not account for the generation of PIN3 asymmetry in response to gravity. Therefore, we investigated whether the polar protein recycling to the plasma membrane is necessary for PIN3 polarization. The fungal toxin brefeldin A (BFA) is a well-established tool for studying trafficking dynamics. In *Arabidopsis thaliana*, BFA inhibits the recycling of PIN proteins from endosomes to the plasma membrane (6, 31, 32). Therefore, we tested the effect of BFA on PIN3 localization. BFA had a stronger effect on the intracellular PIN3 trafficking in gravity-stimulated roots than on nonstimulated roots. After BFA treatment, gravity-stimulated columella cells accumulated PIN3-GFP in so-called BFA compartments, whereas intracellular accumulation occurred only occasionally in nonstimulated seedlings and, when observed, more weakly than in gravity-stimulated seedlings (Fig. 4 *A* and *B*). PIN3-enriched endosomes in nonstimulated roots showed normal motility in the presence of BFA (Fig. S3*E*). These observations reveal that PIN3 trafficking is largely insensitive for BFA in nonstimulated cells, but sensitive in gravity-stimulated cells. Thus, either gravity stimulation generally enhances the rate of PIN3 trafficking through BFA-sensitive endosomes or it promotes recruitment of PIN3 into an alternative, BFA-sensitive targeting pathway. BFA treatments also interfered with PIN3 polarization in response to gravity stimulation and downstream events, including asymmetric auxin distribution and gravitropic bending (Fig. 4 *C–E* and *G*). Thus, BFA-sensitive trafficking mechanisms are required for PIN3 polarization and eventually for other processes important for the gravitropic response.

**Gravity Induces PIN3 Recruitment to the ARF-GEF GNOM-Dependent Polar-Targeting Pathway.** Next, we investigated the molecular target of BFA that mediates its effect on gravity-induced PIN3 polarization. BFA specifically interferes with a subclass of ARF-GEF vesicle transport regulators, including the ARF-GEF GNOM. GNOM regulates the polar localization of basal cargos, such as PIN1 (32), by mediating the constitutive recycling to the basal, but not to the apical, cell side (9, 31). An engineered mutation that renders the GNOM protein insensitive to BFA (32) rescued gravity-induced PIN3 polarization (Fig. 4*F*), auxin redistribution (Fig. 4*H*), and the gravitropic response (32) (Fig. 4*H*), revealing that GNOM is the major target of BFA and that it is a component of the mechanism for gravity-induced PIN3 polarization in gravity-sensing columella cells.

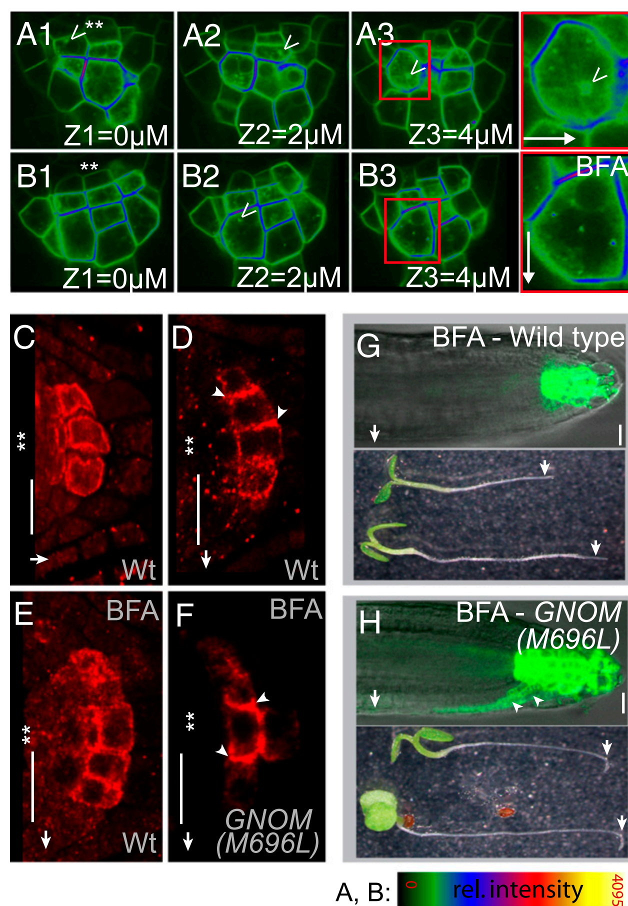


**Fig. 4.** ARF-GEF GNOM regulates PIN3 polarization and asymmetric auxin flow. (*A* and *B*) *Z*-stack analysis of BFA-treated seedlings. Gravity-stimulated seedlings (*A*) display PIN3-GFP accumulation in BFA compartments (see red inset on the right). Nonstimulated seedlings display reduced BFA-mediated PIN3 accumulation (*B*). (*C–F*) Immunolocalization of endogenous PIN3 showed a nonpolar distribution in gravity-sensing columella cells (*C*), but was polarized strongly within 30 min of gravity stimulation (*D*). BFA treatment interfered with PIN3 polarization in wild-type (*E*), but an engineered, BFA-resistant *GNOM*$^{M696L}$ mutant polarized PIN3 upon a gravity stimulation even in the presence of BFA (*F*). Arrowheads mark polarized PIN3 signal (*D* and *F*). (*G* and *H*) BFA treatment interfered with the asymmetric auxin distribution (indirectly visualized by *DR5rev::GFP* in green) and gravitropic response (*G*) of wild-type seedlings. In contrast, *GNOM*$^{M696L}$ mutants displayed BFA-resistant asymmetric auxin distribution and gravitropic response (*H*). Arrowheads indicate asymmetric redistribution of auxin in response to gravity (*H*). Arrows and asterisks indicate the gravity vector and the orientation of the quiescent center, respectively. (Scale bars, 10 μm.)

Although *gnom* knock-out mutants are seedling-lethal and lack roots, weak *gnom* mutant alleles, such as *gnom*$^{R5}$ and *gnom*$^{van7}$, display agravitropic root growth (14). Already without gravity stimulation, PIN3 proteins did not display pronounced plasma membrane localization in columella cells of weak *gnom* (Fig. S3 *F* and *G*), indicating that GNOM function is required for overall PIN3 trafficking. However, prolonged interference with GNOM function affects columella cell identity (10), which might indirectly influence PIN3 trafficking and, therefore, hinder unequivocal interpretation of PIN3 polarization defects in weak *gnom*.

Taken together, our data suggest that gravistimulation induces the recruitment of PIN3 proteins into a BFA-sensitive, GNOM-dependent polar recycling pathway that subsequently generates asymmetric PIN3 localization at the plasma membrane.

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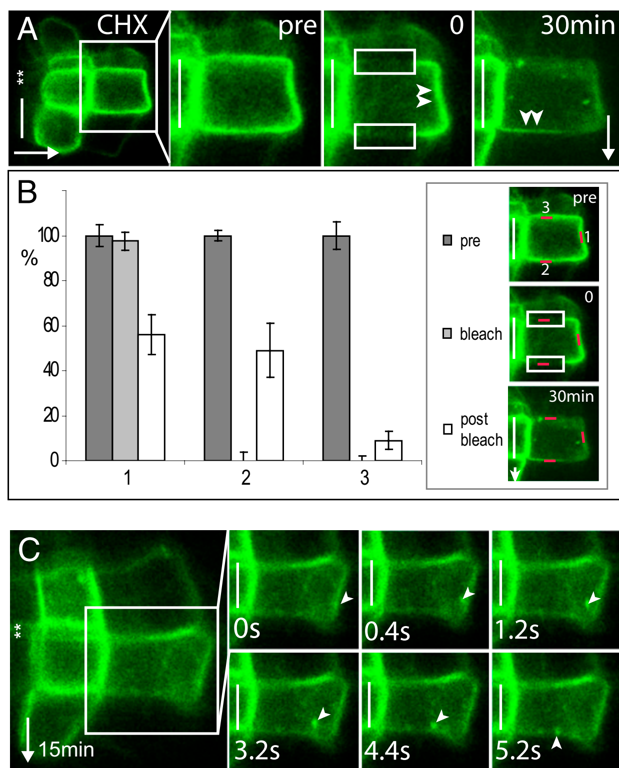


**Fig. 5.** Gravity-induced transcytosis of PIN3. (*A* and *B*) CHX-treated seedlings were photobleached and subsequently stimulated with a 90° gravity stimulus for 30 min (*A*). Arrowheads mark loss of PIN3 signal at the lateral side of the cell and polar recovery in response to gravity at the bottom side of the cell (*A*). Percentage of fluorescent recovery of PIN3-GFP at different sides of the cell after gravity stimulation (*B*). FRAP experiment was done on six seedlings and a representative experiment is shown. (*C*) Seedlings were stimulated for 15 min prior time-lapse analysis. Note the transcytic endomembrane (arrowheads) traveling from the lateral to the new basal cell side. Arrows and asterisks indicate the gravity vector and the position of the quiescent center, respectively. (Scale bars, 5 μm.)

**PIN3 Transcytosis Accounts for Rapid, Gravity-Induced Polarization.** Inhibition of the GNOM-dependent basal recycling pathway results in a basal-to-apical polarity shift of the PIN1 and PIN2 localization and, in principle, alternative ARF GEF-dependent pathways can be used to translocate PIN proteins between different sides of the cell (9). The similar dynamic translocation of polar cargos from one cell side to another via recycling endosomes has been termed transcytosis in animal cells (33). We examined the interesting possibility that gravity-induced PIN3 polarization involves a transcytosis mechanism. To this end, we used fluorescence recovery after photobleaching (FRAP) on PIN3-GFP. Although our previous experiments indicated that the amount of PIN3 secreted within 30 min of gravity stimulation was not significant, we additionally treated seedlings with the protein synthesis inhibitor CHX to ensure that de novo secretion of PIN3-GFP was completely inhibited. PIN3-GFP was bleached on both lateral sides of individual collumella cells and after 30 min of gravity stimulation, a pronounced recovery at the new bottom, but not at the new top, side of the cell was detected (Fig. 5 *A* and *B*). As this recovery cannot be attributed to de novo protein synthesis, this observation suggests that PIN3 is translocated to the bottom cell side from the preexisting pool of proteins in the cell. Concomitantly with the increased PIN3-GFP recovery at the bottom side, the PIN3-GFP signal eventually decreased at the unbleached sides of the cell (i.e., the lateral side) (Fig. 5*B*). These results indicate that PIN3-GFP is translocated to the bottom cell side from the other cell sides, thus demonstrating the gravity-induced transcytosis mechanism for PIN3 polarization.

To visualize the movement of PIN3-GFP endosomes in response to gravity, we stimulated seedlings for 15 min and subsequently performed a PIN3-GFP time-lapse analysis. PIN3-GFP–positive endosomes regularly translocated between two different cell sides, preferentially toward the bottom side of the cell (six positive events out of 30 analyzed movies; in total ≈150 analyzed cells) (Fig. 5*C*). In our analysis, no transcytic endosomes were detected in nonstimulated seedlings (17 analyzed movies with ≈85 cells analyzed in total). This finding might suggest that gravity induces transcytic behavior of a subpopulation of endosomes. To evaluate the statistical significance of this finding we performed a $\chi^2$ test, revealing that the two-tailed *P* value equals 0.0393. By conventional criteria, this difference is considered to be statistically significant. Nevertheless, we cannot completely rule out that transcytic endosomes also occur in nongravity-stimulated columella cells. However, our observation illustrate that PIN3-containing endosomes are trafficking between different cell sides and, hence, can account for PIN3 transcytosis.

Together, the FRAP experiments and movements of endosomes between the cell sides indicate that an endosome-based transcytosis of PIN3 might be induced in response to gravistimulation.

## Conclusions

Here we provide unique insights into the mechanism of gravitropic root growth, in particular into the cellular mechanism of PIN3 auxin transporter polarization in gravity-responding columella cells following gravistimulation. Through a combination of imaging and genetic studies, we show that gravity perception acts upstream of multiple PIN3 trafficking events, including clathrin-mediated PIN3 endocytosis, ARF-GEF–mediated recycling, and endosome-based translocation. Notably, the gravity-induced PIN3 internalization, ARF-GEF–dependent resorting and subsequent polarization shares important analogy to polarized transcytosis in animal cells, where plasma membrane proteins might get internalized at one cell side and translocate to another via recycling endosomes (33). Nevertheless, transcytosis in plants and animals presumably differ in terms of the molecular mechanisms involved (8, 9). In plants, a trans-Golgi network (TGN)-related compartment receives the endocytosed material from the plasma membrane and acts as the early endosome (34). Moreover, the secretory vesicles generated from the TGN in plant cells might move in clusters and eventually fuse with the plasma membrane (35). Hence, the internalization of PIN3 into TGN/early endosomes in response to gravistimulation might lead to the subsequent resorting of PIN3 into transcytic vesicles that move along the gravity vector toward the bottom cell side. This gravity-regulated directional trafficking, potentially in combination with spatially defined fusion with the plasma membrane, might be a plant-specific mechanism to realize PIN3 transcytosis and rapid polarization in response to gravity.

We assume that this process of PIN3 transcytosis to the bottom side of the cell redirects the auxin flux toward the lower side of the root and thus contributes to generating asymmetric auxin accumulation, ultimately leading to asymmetric growth and gravitropic bending. It still remains to be seen whether PIN3 polarization in gravity-sensing columella cells is the initial or sole mechanism to initiate asymmetric auxin distribution during root gravitropic response.

The important open question concerns the exact mechanism for how the gravity perception regulates directional PIN3 transcytosis. Somewhere in the complex mechanism of PIN3 trafficking, the sedimenting statoliths have to provide the directional information that identifies which side of the cell is now the bottom. Whether this identification occurs at the level of the sorting endosomes—where PIN3 is recruited to the polar-recycling pathway, at the level of vesicle fusion with the bottom plasma membrane, or whether the whole polar

recycling trafficking routes are rearranged in response to statolith sedimentation—remain fascinating possibilities for future research.

## Materials and Methods

**Constructs and Growth Conditions.** *DR5rev::GFP* (11), *DR5::GUS* (36), *gnom*$^{R5}$ (14), *GNOM*$^{M696L}$ (32), *PIN3::PIN3-GFP* (27), and *PIN7::PIN7:GFP* (11) have been described previously. PIN3-EosFP was generated with a method analogous to that used to produce the functional PIN3-GFP construct (27). We cloned ≈12 kb of the PIN3 genomic fragment (containing ≈7 kb upstream and 2.5 kb downstream of the coding region) into pBIN19. The Gly-Gly-Gly-(EosFP)-Gly-Gly-Gly-Pro fragment was inserted seamlessly at the end of the first exon with the Counter Selection BAC Modification Kit (Gene Bridges). The system was slightly modified for homologous recombination with the binary vector pBIN19. PIN3-EosFP was transformed via *Agrobacterium tumefaciens* MP90 into *A. thaliana* (L.) Heynh. Plants (ecotype Columbia-0). Kanamycin was used as the selectable marker.

Plants were grown on soil or Murashige and Skoog (MS) plates as described (11) under a 16-h light/8-h dark cycle at 25/20 °C.

**Drug Applications and Experimental Conditions.** Exogenous drugs were applied by incubating 5-d-old seedlings on solid half-strength MS medium supplemented with BFA (stock 50 mM in DMSO) (25/50 μM), CHX (stock 50 mM in DMSO) (50 μM), tyrphostin 23 (stock 50 mM in DMSO) (30/50 μM), or tyrphostin 51 (stock 50 mM in DMSO) (30/50 μM). Control treatments contained an equivalent amount of solvent.

For gravitropism experiments, 5-d-old seedlings were transferred to plates containing a 1-mm layer of MS medium [eventually supplemented with BFA (25 μM)] and were grown in the dark with the plates orientated vertically. Unless indicated differently, a gravity stimulus (90°) was applied for 30 min by positioning plates or slide chambers. A minimum of 40 roots in total were analyzed for each gravity experiment. Asymmetric relocation of *DR5rev::GFP*-expressing seedlings was observed after 3 h and root bending after 6 h of the gravity stimulus. For live-cell analysis of PIN3-GFP, seedlings were gravity stimulated on slides or chambers (Nunc) and subsequently analyzed with vertical confocal microscopy. For all comparisons, at least three independent experiments were done. The gravitropic index was defined as the ratio of the direct distance between the base of the root to the tip and the root length as previously described (37). Statistical significance was evaluated by student's *t* test. $P < 0.05$ was considered as significant, and $P < 0.001$ as highly significant. The vacuolar targeting assay was performed in the dark, as previously described (8). Data were statistically evaluated and visualized with Excel 2003 (Microsoft). For statistical analysis of directional endosomal movements, $\chi^2$ test was performed online as described at http://www.graphpad.com/quickcalcs/chisquared1.cfm.

**Expression and Immunolocalization Analyses.** Whole-mount immunofluorescence was prepared as described (11). For whole-mount immunolocalization in roots, antibodies were diluted as follows: anti-PIN3 (11) and CY3-conjugated anti-rabbit secondary antibodies (Dianova) were diluted 1:100 and 1:600, respectively. For photobleaching experiments, a region of interest was selected for scans with the LCS confocal software 2004 (Leica) FRAP procedure. GFP images before and after scans were collected. EosFP was analyzed as described (9). Photobleached or photoconverted seedlings were subsequently gravity stimulated (in Nunc chamber with solid media) for 30 min and reevaluated via confocal microscopy.

For confocal laser scanning microscopy, a Leica TCS SP2 AOBS with upright microscope stand and an Olympus fluoview FV10 with inverted microcope stand were used. Images were processed in Adobe Photoshop CS2 and assembled in Adobe Illustrater CS2 (Adobe Inc.). Fluorescent signal intensity was analyzed with Image J 1.37v (Rasband) and confocal software (Leica). Data were statistically evaluated with Excel 2003 (Microsoft).

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