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Intracellular Trafficking and Polar Targeting of Auxin Transport Components

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Alice: “Cheshire Cat, would you please tell me which way I ought to go from here?”

Cheshire Cat: “That depends a good deal on where you want to get to”.

Alice: “But I don’t know where I am going”.

Cheshire Cat: “Then it doesn’t matter which way you go”.

(Charles Lutwidge Dodgson)

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Scope and Summary of the Thesis:

Scope: Early plant development launches typically with a relatively simple sketch, resembling a root, stem, and leave like structures. The modesty of plant embryogenesis is followed by a remarkable post embryonic development. Adult plant growth results into shapes that are not predictable by its previous embryonic architecture. Some animals, such as insects, also undergo dramatic postembryonic changes; however, plants are superior in adopting their individual growth to the environmental stress, most likely compensating for their sessile life style. Plants evolved the remarkable ability to redefine the polarity of an already specified tissue, eventually leading to *de-novo* formation of stem cell populations for post embryonic organ formation. The phyto-hormone auxin plays a decisive role in determining and redefining the polarity of plant tissues. Spatial and temporal auxin accumulations (auxin gradients) determine positional cues for the presumptive sites of primordial development. Hence, mechanisms that guide the auxin distribution and, hence, signaling represent a key to understand plant growth. The polar distribution of auxins depends largely on the PIN-FORMED (PIN) auxin efflux carriers that catalyze auxin transport from cell-to-cell. The coordinated polar localisation of PIN proteins at different cell sides determines the direction of auxin flux. Hence, the PIN-dependent auxin distribution affiliates cellular polarizing signals with the polarity of the whole tissue. Moreover, the dynamic nature of the flexible polar PIN localisation regulates plant development by redefining auxin flux and, hence, initiating embryonic and postembryonic developmental programs.

Despite the instrumental importance of cell polarity for plant development, mechanistic insights in establishment of cell polarity are surprisingly limited. The scope of this thesis was to unravel polar targeting mechanisms that guide auxin-dependent plant growth on a molecular and cell biological basis. A special focus was given on directional vesicle transport that determines polarity of auxin influx and efflux carriers.

Summary: Initially, we examined the unique situation of two polarly localised auxin carriers in a single cell, displaying opposite orientations. The auxin influx carrier AUXIN-RESISTANT1 (AUX1) preferentially resides at the apical (upper) cell side in protophloem cells, while PIN1 shows basal (lower cell side) occurrence, enabling us to simultaneously investigate apical and basal polar targeting in plant cells. AUX1 targeting is hypersensitive to actin depolymerisation and stabilization, suggesting strict

actin cytoskeletal requirements for apical AUX1 localisation (chapter 2). Interestingly, apical PIN2 localisation in the epidermis also shows higher sensitivity to actin depolymerisation compared to basal PIN2 targeting in the lower cortex cells (chapter 4). However, both apical and basal PIN targeting largely depend on the intact actin cytoskeleton, suggesting a general requirement for directional vesicle transport along actin filaments (chapter 4). PIN1 and AUX1 show actin-dependent constitutive recycling between the plasma membrane and an endosomal compartment; however, while PIN1 cycling requires the activity of the vesicle transport regulator ARF-GEF GNOM, AUX1 recycling and intracellular targeting is independent of GNOM (chapter 2). Hence, apical AUX1 and basal PIN1 targeting are molecularly distinct and get facilitated by two sovereign pathways (chapter 2, chapter 4). Independent targeting of auxin influx and efflux carrier at a single cell level appears to have functional relevance for fine tuning polar auxin transport during particular developmental events.

We illustrated two polar competent proteins to utilize independent targeting pathways in the same cell, raising the question how a particular polar cargo gets recruited to different cell sides. Notably, we found apical and basal PIN targeting to be also molecularly distinct by ARF/ARF-GEF utilization (chapter 3; chapter 4). Endosomal ARF GEF GNOM regulates basal PIN targeting, whereas, apical targeting is independent of GNOM (chapter 3). Moreover, pharmacological inhibition of GNOM function by brefeldin A (BFA) interferes with basal but not apical PIN targeting and, subsequent, leads to a preferential basal-to-apical PIN polarity shift (chapter 3). Live cell imaging of photoactivateable PIN2-EosFP reveals ARF GEF dependent translocation of the same PIN2 molecules from one cell side to the other via intracellular sorting, indicating that the distinct apical and basal targeting pathways are interconnected (chapter 3). This process is reminiscent to polar cargo translocation in animal epithelial cells termed transcytosis. We illustrated polar transcytosis to be operational in untreated plant cells and propose that a GNOM dependent PIN transcytosis triggers important developmental events, such as apical-basal axis formation during embryogenesis and postembryonic de-novo organ formation (chapter3).

The rate and the directional output of the ARF GEF dependent PIN polarity alterations are cell type dependent, in most severe cases, determining the directional output of the polarity alteration. Beside cell type determinants, the PIN transcytosis largely depends on the polar cargo itself (chapter 4). Notably, the ARF GEF dependent apical-basal transcytosis

pathways appear to be specific for PIN proteins, because other polar cargos, such as the AUX1, do not get recruited (chapter 4), confirming the assumptions for independent AUX1/PIN targeting pathways. Similar to intracellular AUX1 targeting, intracellular and apical PIN2 targeting in root epidermal cells are independent of GNOM, but nevertheless, the data on PIN2 targeting suggest ARF dependency (chapter 4), indicating the involvement of a BFA resistant ARF GEF in apical PIN trafficking. However, the GNOM homolog GNOM-LIKE1, which has been shown to be BFA resistant, is unlikely to be involved in apical PIN targeting and transcytosis (chapter 4). Apical and lateral PIN2 targeting requires the activity of other BFA resistant and sensitive ARF GEFs, respectively, illustrating a complex regulatory network for polar PIN targeting to different cell sides (chapter 4).

Next we aimed to unravel additional molecular components for the plant transcytosis pathways. Several SORTING NEXINs (SNX) are retromer complex components and have been reported to regulate polar transcytosis in animal epithelial cells. The putative plant retromer components AtSNX1 and AtVPS29 have been suggested to be involved in polar PIN targeting. However, SNX1 and VPS29 are not directly involved in apical/basal targeting, recycling and transcytosis of PIN proteins (chapter 3, chapter 5). In contrast, we illustrate that PIN protein stability is reduced in *snx1* and *vps29* mutants (chapter 5). To further address plant retromer involvement in PIN targeting, we characterized lytic degradation in plants, using a novel drug independent approach for visualizing PIN targeting to the lytic vacuole (chapter 5). Vesicle transport along the actin cytoskeleton regulates vacuolar targeting of PIN2 by utilizing novel ARF GEF and phosphatidylinositol-3-kinase dependent pathways (chapter 5). We unravelled SNX1/VPS29 to have a gating function at the pre vacuolar compartment and illustrate that SNX1 is required for the restriction of the temporal degradation of PIN2 proteins in lytic vacuoles during gravitropic response (chapter 5). Collectively, our data suggest that ARF GEF and SNX1-dependent vacuolar targeting of PIN proteins is required for the fine tuning of polar auxin transport during plant development.

Collectively, we provide the first mechanistic insights into intracellular targeting of PIN proteins, addressing their polar distribution, polarity alterations and protein stability; moreover, we illustrate the functional importance of these processes for the plant development. Directional vesicle transport regulates the PIN activity at the plasma membrane via various sorting events, determining the direction and rate of

the polar auxin transport and, thus, contributes substantially to auxin dependent shaping of the individual plantlet.

Chapter 1

Introduction and concluding remarks:

Polar Targeting and Endocytic Recycling in Auxin-Dependent Plant Development

Polar Targeting and Endocytic Recycling in Auxin-Dependent Plant Development

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Key Words

trafficking, endocytosis, polar auxin transport, PIN proteins

Abstract

Plant development is characterized by a profound phenotypic plasticity that often involves redefining of the developmental fate and polarity of cells within differentiated tissues. The plant hormone auxin and its directional intercellular transport play a major role in these processes because they provide positional information and link cell polarity with tissue patterning. This plant-specific mechanism of transport-dependent auxin gradients depends on subcellular dynamics of auxin transport components, in particular on endocytic recycling and polar targeting. Recent insights into these cellular processes in plants have revealed important parallels to yeast and animal systems, including clathrin-dependent endocytosis, retromer function, and transcytosis, but have also emphasized unique features of plant cells such as diversity of polar targeting pathways; integration of environmental signals into subcellular trafficking; and the link between endocytosis, cell polarity, and cell fate specification. We review these advances and focus on the translation of the subcellular dynamics to the regulation of whole-plant development.

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DEVELOPMENTAL INTRODUCTION

Animals and plants evolved basic biological differences that characterize their survival strategies. Animals developed elaborated sensory and locomotory capacities that enable complex behavioral responses, such as the fight-or-flight response, to overcome environmental stress. In contrast, during their evolution plants emphasized increased physiological tolerance and phenotypic plasticity. These different life strategies are also adequately reflected in the various ways in which animals and plants establish their body architecture. Whereas during embryogenesis animals are already defining their adult shape to a large extent, in plants this early developmental phase just sketches a basic body plan, and the final shape of a plant will be largely defined by an elaborate postembryonic development (Weigel & Jürgens 2002). To achieve this developmental plasticity, plants maintain permanent populations of stem cells (meristems) at the growing root and shoot apices and are able to redefine the developmental programs as well as the polarity of already specified tissues. Thus, plants can sustain and regulate their growth rate, can postembryonically form new organs, and possess a high capacity for tissue regeneration (Steeves & Sussex 1989, Weigel & Jürgens 2002). Different animal species also retain these capabilities to some extent; however, plants are far superior in utilizing these mechanisms for individually shaping their body according to the demands of the environment. The plant signaling molecule auxin determines many aspects of this flexible plant development. Auxin acts as a prominent signal, providing, by its local accumulation in selected cells, a spatial and temporal reference for changes in the developmental program (Reinhardt et al. 2000, Friml 2003, Leyser 2006, Esmon et al. 2006, Tanaka et al. 2006, Dubrovsky et al. 2008). Auxin is distributed through tissues by a directional cell-to-cell transport system, termed polar auxin transport, that depends on specific auxin carrier proteins (**Figure 1**) (Benjamins et al. 2005, Blakeslee et al. 2005, Kramer & Bennett 2006, Vieten et al. 2007). Auxin efflux carriers of the PIN-FORMED (PIN) family (Gälweiler et al. 1998, Luschnig et al. 1998, Chen et al. 1998, Utsono et al. 1998, Petrášek et al. 2006) show a polar subcellular localization that correlates with and determines the

Polar auxin transport: the directional transport of the plant hormone auxin from cell to cell

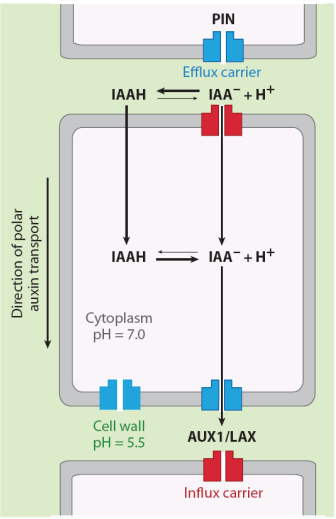

Figure 1

The chemisosmotic hypothesis: far ahead of its time! Rubery & Sheldrake postulated in mid-1970s the so-called chemiosmotic hypothesis for directional intercellular auxin movement (Rubery & Sheldrake 1974 and, independently, Raven 1975). Accordingly, the auxin indole acetic acid (IAAH) is largely protonated at the lower pH of the cell wall and can pass through the plasma membrane into the cell. In the higher-pH cytosol, part of the IAAH is deprotonated, and the resulting charged IAA^- is largely membrane impermeable and requires transporter activity to exit the cell. The localization of the PIN-FORMED (PIN) auxin efflux carrier at the plasma membrane determines the auxin exit site from an individual cell. Coordinated polar localization of PINs in a given tissue hence determines the direction of cell-to-cell auxin transport. AUX1/LAX1 denotes auxin influx carriers AUXIN RESISTANT1/LIKE AUX1.

direction of auxin flow through tissues (Friml et al. 2004, Wisniewska et al. 2006). In plants, polarities of tissue and of individual cells are closely connected by the flow of auxin (Sauer et al. 2006), and the cell biological processes depending on vesicle trafficking and polar targeting have an immediate developmental output related to auxin-mediated signaling. At the level of polar auxin transport, many developmental and environmental signals are integrated. By rearranging the subcellular localization of PIN auxin efflux carriers, such signals influence auxin-dependent patterning and contribute substantially to the adaptive and flexible nature of plant development.

Our aim is to review recent advances on subcellular trafficking and polar targeting in plants and to highlight links with physiology and development. A special focus is given to auxin-dependent regulation of development because this area is intimately linked to endocytic recycling and polar targeting. Most of these concepts were formulated on the basis of studies in the model plant *Arabidopsis thaliana*; nonetheless, they seem to apply to a large extent to higher plants in general.

Recycling: membranes and other molecules recycle from intracellular endocytic compartments back to the plasma membrane

Tight junctions: anchored protein complexes forming a physical barrier between polar domains; limit lateral diffusion and are involved in polarity establishment and maintenance in animal epithelial cells

POLAR TARGETING

The establishment and maintenance of cell polarity are central themes of developmental and cell biology because individual cell polarities, transmitted by cell divisions, are translated into tissue and organ polarity and, ultimately, shape. In addition, cell polarity plays a key role in directional signaling and intercellular communication.

At the level of individual cells, polarity is typically reflected by the asymmetric distribution of intracellular components that can form functionally and/or morphologically distinct domains (Bonifacino & Lippincott-Schwartz 2003). Mechanisms for generating or maintaining cell polarity have been extensively studied in different model organisms, such as worms, flies, mammals, and yeasts (e.g., Knoblich 2000, Irazoqui & Lew 2004, Margolis & Borg 2005, Nance 2005). Animal epithelial cells are a favorite model system because their plasma membrane harbors two distinct domains that are separated by tight junctions: an apical domain facing the lumen and a basolateral domain (Mostov et al. 2003, Janssens & Chavrier 2004). These protein-based barriers in the membrane prevent lateral diffusion of proteins and lipids

trans-Golgi network (TGN): the main sorting compartment of the secretory pathway in eukaryotic cells; may act as an early-endosomal compartment in plants

between the two distinct polar domains, maintaining the distribution of various polar-competent proteins. Researchers have identified numerous polar cargos that reside in a cell-line-specific manner preferentially at the apical and/or basolateral plasma membranes in polarized epithelial cells. Apical and basolateral components are recruited differentially by the targeted delivery of membrane and secretory proteins to these domains as a result of three processes. (*a*) Newly synthesized proteins are sorted in the trans-Golgi network (TGN) into carrier vesicles that specifically deliver them to the apical surface or the basolateral surface. (*b*) Some proteins are selectively retained at the plasma membrane. (*c*) Proteins that are not retained are rapidly endocytosed and either recycled back through recycling endosomes or, alternatively, delivered to a different, polar plasma membrane domain by a process called transcytosis (Rodriguez-Boulan et al. 2005).

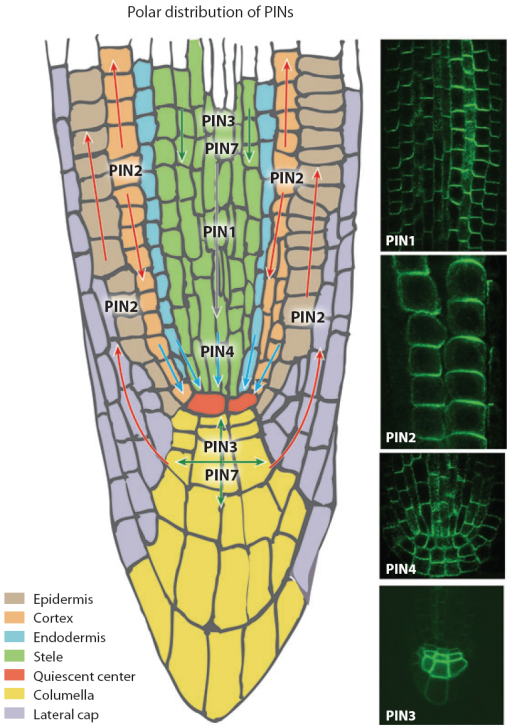

Figure 2

Patterns of PIN protein localization in the *Arabidopsis* root tip. Schematic and immunolocalizations of PIN proteins in the *Arabidopsis* root tip. Arrows indicate polar PIN localization at the plasma membrane, illustrating cell type–dependent decisions in the PIN polar localization. Note the differential PIN2 targeting in the epidermis (apical) and young cortex (basal) cells.

Passengers and Destinations: Polar Cargos and Polar Domains

Even though in no other kingdom is the relation between individual cell polarity and macroscopic patterning as prominent as in plants, knowledge on cell polarity and mechanisms of targeted cargo delivery is still lacking in plants. Most of our understanding on polar targeting has been gained by study of the polar delivery of auxin efflux carriers from the PIN family (**Figure 2**). PIN proteins have emerged in recent years from genetic studies in *A. thaliana* as key regulators of a plethora of auxin-mediated developmental processes, including axis formation in embryogenesis (Friml et al. 2003b), postembryonic organogenesis (Okada et al. 1991, Benková et al. 2003, Reinhardt et al. 2003, Heisler et al. 2005), root meristem maintenance (Friml et al. 2002a, Blilou et al. 2005), vascular tissue differentiation and regeneration (Gälweiler et al. 1998, Sauer et al. 2006, Scarpella et al. 2006), and tropic growth (Luschnig et al. 1998, Friml et al. 2002b).

PIN proteins act as mediators of the auxin efflux from cells (Petrášek et al. 2006) and have different subcellular distributions—including apolar, basal, apical, and lateral plasma membrane localizations—depending on the PIN protein as well as the cell type (Wisniewska et al. 2006). The most typical are basal (root tip–facing) localization of the PIN1 protein in the inner cells of both shoots and roots, apical (shoot apex–facing) localization of PIN2 in the

root epidermis and lateral root cap cells, and lateral localization of PIN3 at the inner side of shoot endodermis cells (Gälweiler et al. 1998; Müller et al. 1998; Friml et al. 2002b, 2003a).

Other components of auxin transport, such as the auxin influx carrier AUXIN RESISTANT1/LIKE AUX1 (AUX1/LAX) (Bennett et al. 1996, Yang et al. 2006, Swarup et al. 2008) and multiple drug resistance/P-glycoprotein (MDR/PGP) transporters (Geisler et al. 2005, Terasaka et al. 2005), are also localized in a polar manner in some cells while being symmetrically localized in most cells (Mravec et al. 2008). For example, AUX1 localizes to the apical side of protophloem cells opposite to PIN1 or to the same side as PIN1 in the shoot apical meristem (Swarup et al. 2001, Reinhardt et al. 2003). In contrast, PGP4 has a basal or an apical localization in root epidermal cells (Terasaka et al. 2005).

In addition to components of the auxin transport, other polar cargos in plants, including transporters for boron (BOR1 and BOR4) and for silicon in rice (LSI1 and LSI2), have been identified. Such cargos are localized at either the inner or the outer lateral sides of cells, as well as the regulator of anisotropic expansion, COBRA, which is similarly polarly targeted to both longitudinal cell sides (Roudier et al. 2005; Takano et al. 2005; Ma et al. 2006, 2007; Miwa et al. 2007). The PLEIOTROPIC DRUG RESISTENCE (PDR)-type transporter for the auxin-like compounds PIS1/PDR9 resides at the outer lateral side of root epidermis cells. The lateral cargo POLAR AUXIN TRANSPORT INHIBITOR-SENSITIVE1 (PIS1), the basal cargo PIN1, and the apical cargo PIN2 have been simultaneously visualized in the same cells, highlighting that plant cells are able to maintain at least three polar domains within a single cell (Růžička et al. 2008). Future studies will address whether epidermal root cells are potent to maintain, besides the apical, basal, and outer lateral domains, an additional inner lateral polar domain. Nonetheless, although apical-basal targeting in plants and apical-basolateral delivery in animals can reflect a comparable polar competence among the divergent kingdoms, the simultaneous delivery of lateral cargos hints at a more complex situation for cell polarity in plant cells that may once again stress the flexibility and enormous importance of cell polarity regulation in plants (**Figure 3**).

Endocytosis: the uptake of material into a cell by the formation of a membrane-bound vesicle

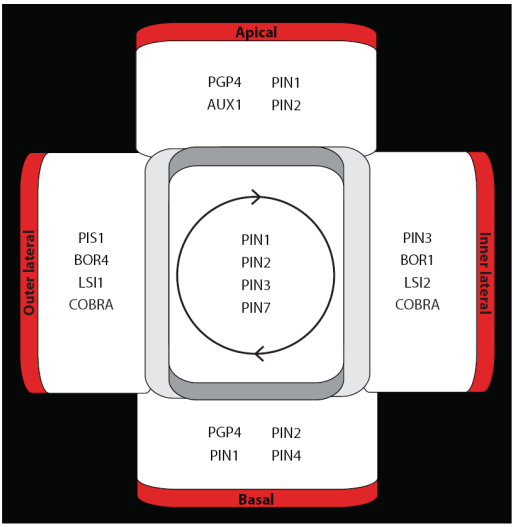

Figure 3

The black box of plant polarity. A schematic representation of various polar cargos in plant cells. Plants are competent to deliver cargos to apical, basal, inner, and outer polar domains. Apical cargos include PGP4 (root epidermis cells), AUX1 (root protophloem cells), PIN1 (epidermal cells of shoot apex and embryonal protoderm cells), and PIN2 (root epidermis and older cortex cells). Basal cargos encompass PGP4 (root epidermis cells), PIN1 (e.g., root stele), PIN2 (young cortex cells), and PIN4 (in the proximal part of the root meristem). Outer lateral cargos are represented by, e.g., PIS1 (root epidermis), BOR4 (root epidermis), LSI1 (root exodermis and endodermis cells), and COBRA (root epidermis). Inner lateral polarity can be defined by PIN3 (shoot endodermis and root pericycle), BOR1 (root pericycle cells), LSI2 (root exodermis and endodermis cells), and COBRA (root epidermis). Moreover, several PIN cargos undergo rapid polarity alterations (depicted in the *middle*), including the establishment of basal localization of PIN1 during embryogenesis or lateral root development, an apical-to-basal polarity shift of PIN7 during embryogenesis in suspensor cells, a basal-to-apical shift in upper cortex cells of PIN2, and dynamic relocation of PIN3 to the bottom sides of root cap cells after gravity stimulation.

Transcytosis: the dynamic translocation of the same molecules from one distinct plasma membrane domain to another via recycling endosomes

Basal polarity: polarity of the lower cell side, the polar plasma membrane domain that faces the root apex

Apical polarity: polarity of the upper cell side, the polar plasma membrane domain that faces the shoot apex

Inner lateral polarity: polarity of the inner periclinal cell side, which points away from the body surface

Outer lateral polarity: polarity of the outer periclinal cell side, which points to the body surface

Tickets to Go or to Stay: Polar Targeting Signals

In animal systems, polar cargo proteins carry signals that determine their residence at different polar domains. These signals may be a combination of plasma membrane retention, internalization, and polar sorting signals (Dugani & Klip 2005, Rodriguez-Boulan et al. 2005). In plants, different polar cargos such as PIN1, PIN2, and PIS1 localize to different polar destinations in the same cell type, suggesting polarity determinants in the protein sequence itself. Moreover, an insertion of green fluorescent protein (GFP) at a specific position within the middle hydrophilic loop causes PIN1 localization to shift to the oposite side of the cell compared with wild-type PIN1 (Wiśniewska et al. 2006). These results demonstrate the presence of polarity signals in the sequence of polar cargos, but detailed insight is still lacking. Polarity signals probably decide to recruit PINs to the distinct apical and basal targeting machineries that are related to phosphorylation sides, because the Ser/Thr protein kinase PINOID (PID) (Friml et al. 2004) as well as the protein phosphatase 2A (PP2A) (Michniewicz et al. 2007) act on PIN phosphorylation and play a decisive role in the apical-versus-basal targeting of PIN proteins. Loss of the PID function causes an apical-to-basal shift in the PIN polarity corresponding with defects in embryo and shoot organogenesis (Christensen et al. 2000, Benjamins et al. 2001, Friml et al. 2004). Accordingly, PID gain of function results in an opposite basal-to-apical PIN polarity shift, leading to auxin depletion from the root meristem and collapse of the root growth (Friml et al. 2004). Similar phenotypes, including the basal-to-apical shift of PIN polarity, can be observed in the loss-of-function mutants of the A regulatory subunits of PP2A (Michniewicz et al. 2007). Importantly, PID directly phosphorylates the hydrophilic loop of PIN proteins, and PP2A antagonizes this action (Michniewicz et al. 2007).

A possible scenario may be that phosphorylated PIN proteins are preferentially recruited into the apical pathway, whereas dephosphorylated PINs become a substrate of the basal targeting pathway (**Figure 4**). This model incorporates important features of mammalian epithelial cells, in which cargos are phosphorylated to influence their polar delivery (Casanova et al. 1990). Importantly, phosphorylation-dependent PIN targeting provides a means for any signaling pathway upstream of PID and PP2A activities to modulate PIN polar targeting and thus directional auxin fluxes. Different relative expression levels of PID and PP2A in various cell types in combination with divergent

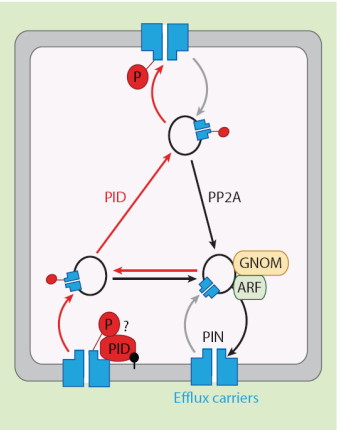

Figure 4

Contribution of PIN-FORMED (PIN) phosphorylation to the decision on the PIN polar distribution. PINOID (PID)-dependent phosphorylation of PIN proteins may affect affinity to distinct apical and basal targeting pathways. An increase in PID kinase or a decrease in protein phosphatase 2A (PP2A) activities leads to a basal-to-apical PIN polarity shift. On the contrary, increased PP2A activity counteracts the PID effect and leads to preferential GNOM-dependent basal PIN targeting. The place of PID and PP2A action is not entirely clear, but PID and PP2A are also partially associated with the plasma membrane. ARF denotes adenosyl ribosylation factor.

phosphorylation sites (some of which would be phosphorylated more or less efficiently) would explain how both PIN-specific and cell type–specific signals are integrated to determine the polar localization of the given PIN protein into a given cell type. The regulation of PID kinase may also be connected with phospholipid signaling. The plant 3-phosphoinositide-dependent kinase 1 (PDK1) binds PID in vitro and increases PID kinase activity (Zegzouti et al. 2006). The involvement of phosphorylation events for the polar delivery of other cargos besides PINs has not been thoroughly addressed. Outer lateral delivery of PIS1 seems to occur independently of the PID activity, but comparable information for other polar cargos is missing.

Extensive work in the coming years is expected to focus on the identification and thorough characterization of polar targeting signals for different polar cargos in plants. The other crucial issues that are completely unknown in plants concern where and how the polar targeting signals are recognized as well as where and how the polar cargos are sorted.

Staying at the Station: Retention at the Polar Domains

Despite the pronounced importance of polar localization of proteins in plant cells for plant development, mechanisms for this phenomenon are still ill defined. So far no indications exist for anything analogical to tight junctions, and we lack even fundamental knowledge of how polar-competent cargos are kept in their polar domains.

Cytoskeleton- and membrane sterol–dependent constitutive endocytosis and targeted recycling may be involved in maintaining the localization of proteins localized in their polar domains. All these cellular components and processes are required for localization of different cargos. Both basal PIN1 localization and even more apical AUX1 localization are sensitive to the disruption of the actin cytoskeleton, leading to internalization and loss of polar localization (Kleine-Vehn et al. 2006). In contrast, disruption of microtubules affects only indirectly the localization of AUX1 and PIN proteins that is observed only when the overall cell morphology is altered (Kleine-Vehn et al. 2006). In contrast, intact microtubules are required to maintain the outer lateral localization of PIS1; following its disruption, PIS1 is found predominantly at apical and basal positions (Růžička et al. 2008).

Polar localization of PIN and AUX1 proteins as well as auxin signaling depend on the sterol composition of plasma membranes (Souter et al. 2002, Grebe et al. 2003, Willemsen et al. 2003, Kleine-Vehn et al. 2006). *Arabidopsis* plantlets defective in the *STEROL METHYLTRANSFERASE1* (*SMT1*) gene, which is involved in sterol biosynthesis and affects membrane sterol composition, have cell polarity defects, including impaired polar localization of PIN proteins and AUX1 (Willemsen et al. 2003, Kleine-Vehn et al. 2006). Furthermore, sterols and PIN proteins have overlapping subcellular trafficking pathways (Grebe et al. 2003). Detergent-resistant, sterol-enriched plasma membrane microdomains, sometimes called lipid rafts, are important for various types of plasma membrane–based signaling processes and are present in higher plants as well (reviewed in Bhat & Panstruga 2005). There are indications that PIN and PGP proteins are directly associated with these structures (Titapiwatanakun et al. 2008), but what the functional relevance could be of such associations remains to be established.

In conclusion, despite the obvious requirements of cytoskeleton and sterol composition for polar localization of various cargos, it is still unclear whether or eventually in which cases they are involved in keeping cargos at their polar positions or whether they play a role in the polar delivery of these proteins to the plasma membrane.

How to Get There: Polar Targeting Pathways

The existence of diverse polar cargos with various polar targeting signals implies a diversified

Sterol: plant sterols are amphiphilic molecules and vital constituents of all membranes, including the plasma membrane

network of distinct polar targeting pathways. Indeed, for example, AUX1 and PIN1 polar delivery occurs by two distinct targeting machineries with different molecular requirements and different sensitivities to inhibitors of cellular processes (Dharmasiri et al. 2006, Kleine-Vehn et al. 2006).

An important factor for the delivery of PIN proteins to the plasma membrane is an endosomal regulator of the vesicle budding, GNOM, which encodes a guanine nucleotide exchange factor for adenosyl ribosylation factors (ARF GEF) (Shevell et al. 1994; Geldner et al. 2001, 2003). In *gnom* (also designated *emb30*) mutant embryos, the coordinated polar localization of PIN1 is impaired (Steinmann et al. 1999), seemingly the result of a failure to establish the initial basal localization of PIN1 at the globular stage (Kleine-Vehn et al. 2008a). Also, in the postembryonic roots, GNOM function seems to be crucial for basal targeting, whereas apical localization of PINs or AUX1 is unaffected in *gnom* mutants (Kleine-Vehn et al. 2006, 2008a). Collectively, these studies demonstrate that apical cargos utilize a targeting pathway that is molecularly distinct from that used by basally localized PIN proteins (Kleine-Vehn et al. 2006, 2008a). In addition, outer lateral PIS1 targeting appears to differ fundamentally from apical and basal pathways because PIS1 polar localization does not involve any known molecular components of apical/basal targeting, such as GNOM or PINOID (Růžička et al. 2008). Although apical and basal PIN targeting appears to be interconnected and, thus, to be used alternatively by PIN proteins, the relation between apical/basal and outer lateral polar targeting needs to be unraveled.

In summary, genetic and pharmacological interference with different cellular processes as well as the simultaneous localization of cargos to the apical, basal, and outer lateral domains in single cells strongly suggest that there are at least three distinct polar targeting mechanisms in plants. However, molecular insight into these pathways remains very limited.

Adenosyl ribosylation factor (ARF): a class of small GTPases; a regulator of clathrin- and COPI-dependent vesicle budding

Guanine nucleotide exchange factor (GEF): induces GDP-to-GTP exchange and hence the activation of small GTPases

Brefeldin A (BFA): a specific inhibitor of some ARF GEFs

ENDOCYTIC RECYCLING IN PLANT CELLS

The Back and Forth: Constitutive Endocytic Recycling of Plasma Membrane Proteins

The internalization of proteins from the plasma membrane is a critical event for all eukaryotic cells. Whereas many internalized molecules are degraded in the lysosomal/vacuolar pathway, other cell surface proteins and molecules undergo sequential rounds of recycling back to the plasma membrane. Eukaryotic cells possess the remarkable ability to turn over the entire plasma membrane on an hourly basis (Tuvim et al. 2001). As such, endocytic recycling is a key for the regulation of the cell surface identity and contributes to rapid cellular responses to intrinsic and extrinsic cues. Regarding the fundamental importance of endocytic recycling, various integral plasma membrane proteins, such as signaling components and transporters, appear to display recycling events to the plasma membrane in plants.

Pharmacological inhibitors have been valuable tools for unraveling the internalization of plant proteins to endosomal compartments and subsequent recycling back to the plasma membrane (Carter et al. 2004). The fungal toxin brefeldin A (BFA) interferes with various vesicle trafficking processes in cells and specifically targets ARF GEFs. Cytosolic GDP-bound ARF proteins are inactive and become recruited to the target membrane by ARF GEF–dependent GDP-to-GTP exchange. ARF proteins play an important role in the formation of vesicle coats required for their budding and cargo selection in different subcellular compartments. BFA is a noncompetitive inhibitor that stabilizes ARF/ARF GEF intermediates and freezes both proteins inactively at the place of action (reviewed by Donaldson & Jackson 2000). In cultured cells of tobacco, BFA interferes with ARF GEF–dependent endoplasmic reticulum (ER)-to-Golgi trafficking, leading to ER-Golgi hybrids (Ritzenthaler et al. 2002). In contrast, in *Arabidopsis*, this process is catalyzed by the

BFA-resistant ARF GEF GNOM-LIKE1 (GNL1) (Richter et al. 2007). The prominent BFA target in *Arabidopsis* is the endosomal ARF GEF GNOM, which mediates mainly the endosomal recycling to the plasma membrane, whereas endocytosis from the plasma membrane seems to remain operational (Geldner et al. 2003). By this differential effect of BFA on exocytosis and endocytosis in *Arabidopsis*, plasma membrane proteins are internally accumulated into so-called BFA compartments (Geldner et al. 2001, 2003).

In *Arabidopsis* seedlings, following BFA treatments PIN1 rapidly disappears from the plasma membrane and simultaneously aggregates in BFA compartments (Steinmann et al. 1999). This process is fully reversible because BFA removal causes PIN proteins to relocalize to their original position at the plasma membrane (Geldner et al. 2001). Both the internalization and the recovery after washout also occur in the presence of protein synthesis inhibitors, indicating that they are not de novo–synthesized proteins but involve continuous endocytosis and recycling of the same PIN molecules (Geldner et al. 2001). The utilization of a green-to-red photoconvertible fluorescent reporter (EosFP) directly visualizes the internalization of PIN proteins and their subsequent recycling to the plasma membrane (Dhonukshe et al. 2007a). These findings indicate an operational constitutive cycling mechanism in plant cells.

BFA-sensitive subcellular dynamics have been demonstrated for a number of plasma membrane proteins, including, for instance, the aquaporin PIP2, the brassinosteroid receptor BRI1, the plasma membrane H^+-ATPase, the stress-responsive plasma membrane protein Lti6a, and the auxin influx carrier AUX1 (Geldner et al. 2001; Grebe et al. 2002, 2003; Russinova et al. 2004; Paciorek et al. 2005). This BFA sensitivity may reflect a PIN-like mechanism of constitutive endocytosis and recycling, as is seemingly the case for many intrinsic plasma membrane proteins, and has been demonstrated for BRI1, whose endocytic recycling rate may be regulated by heterodimerization with the associated kinase BAK1 (Russinova et al. 2004). However, endocytic recycling is not necessarily accompanied with BFA-sensitive trafficking, as exemplified by both polar and nonpolar delivery of AUX1 to the plasma membrane that is largely insensitive to BFA (Kleine-Vehn et al. 2006). Another example for a recycling plasma membrane protein is the inwardly directed K^+ channel KAT1. The hormone abscisic acid, which controls ion transport and transpiration in plants under water stress, may trigger the selective endocytosis of the KAT1 in epidermal and guard cells, leading to changes in K^+ channel activities at the plasma membrane. Abscisic acid treatment sequesters the K^+ channel within an endosomal membrane pool that recycles back to the plasma membrane within hours (Sutter et al. 2007).

Despite the mostly indirect evidence (based mainly on BFA-sensitive targeting), the number of plant proteins that constitutively recycle at different rates from and to the plasma membrane is constantly growing. In fact, it seems rather difficult to find an intrinsic plant plasma membrane protein that would not undergo BFA-sensitive or BFA-insensitive constitutive recycling. However, besides the fact that almost all plant plasma membrane proteins appear to recycle between the plasma membrane and some intracellular compartments, the mechanisms underlying their differential endocytosis and recycling are still not well characterized.

Getting Away: Endocytosis in Plant Cells

Endocytosis occurs at the cell surface and is characterized by membrane invagination and pinching off at the plasma membrane, ultimately leading to closed membrane vesicles in the cytoplasm. These mechanisms facilitate the absorption of material from the outside of the plasma membrane and have been studied mainly in animal cells. Several distinct pathways for endocytosis have been unraveled; among these are the relatively well-defined processes of macropinocytosis, clathrin-mediated endocytosis, and caveolae-mediated endocytosis

Exocytosis: the process by which materials in the vesicles are secreted from a cell when the vesicle membrane fuses with the plasma membrane

BFA compartment: BFA treatment–induced mixture of aggregated vesicles in *Arabidopsis* that consists of endosomal and TGN-derived structures in the core and that becomes surrounded by aggregating Golgi stacks

(Pelkmans & Helenius 2003, Cheng et al. 2006, Benmerah & Lamaze 2007). Macropinocytosis is a less specific invagination of the cell membrane, resulting in the pinching off of vesicles (Pelkmans & Helenius 2003). In contrast, clathrin-dependent endocytosis and caveolae-dependent endocytosis regulate receptor-mediated internalization of specific cargos (Benmerah & Lamaze 2007).

In plants, the existence of endocytosis has been regarded with skepticism, and there have been decades of controversial debates as to whether the high turgor of plant cells renders the plant plasma membrane unsuitable for invagination and subsequent internalization (Saxton & Breidenbach 1988, Gradmann & Robinson 1989). Experimental results have often settled the theoretical discussions: Electrondense as well as lipophilic tracers have been taken up into plant cells (Robinson & Hillmer 1990, Bolte et al. 2004), endocytic (clathrin-coated) vesicles have been detected at the ultrastructure level (reviewed in Holstein 2002, Paul & Frigerio 2007), and numerous proteins have been found to be internalized from the plasma membrane (Geldner et al. 2001; Paciorek et al. 2005; Takano et al. 2005; Dhonukshe et al. 2006, 2007a; Robatzek et al. 2006), even in high-turgor guard cells (Meckel et al. 2004). However, the underlying mechanism of endocytosis in plants has remained unclear until recently (Pérez-Gómez & Moore 2007). There were growing lines of evidence that the endocytosis mechanism involving the coat protein clathrin is operational in plant cells. The deciphering of several plant genomes revealed that homologs to mammalian proteins of the clathrin coat (e.g., clathrin heavy chain, clathrin light chain, adaptor protein (AP)2 subunits, and AP180) and downstream effectors (e.g., epsin, dynamin, auxilin, heat shock cognate 70, and synaptojanin) are encoded in plant genomes (Hirst & Robinson 1998, Holstein 2002, Paul & Frigerio 2007). Additionally, electron microscopy has detected different stages of clathrin-coated vesicle formation at plasma membranes of different plant species (reviewed in Holstein 2002, Paul & Frigerio 2007). Finally, genetic and pharmacological interference with clathrin-dependent processes in plants blocks internalization of PINs and other plasma membrane proteins (Dhonukshe et al. 2007a). Altogether, these studies have demonstrated operational clathrin-dependent endocytosis in plants and have identified the endogenous cargos of this process. Clathrin-dependent endocytosis seems to be remarkably evolutionarily conserved because mammalian cargos of this pathway, such as the transferrin receptor, are internalized by this mechanism in plant cells (Ortiz-Zapater et al. 2006). Moreover, because the internalization of all tested cargos, including general endocytic tracers, requires clathrin, clathrin-dependent endocytosis seems to constitute the predominant pathway for the internalization of numerous plasma membrane–resident proteins in plant cells.

It remains to be seen whether clathrin-independent pathways are operational in plant cells. Pathways for sterol-dependent, caveolae-mediated endocytosis are unlikely to exist because caveolae-like components have not been identified in plants. However, there are indications suggesting the involvement of sterols in endocytosis or endocytic recycling in plants. Polar PIN protein localization is affected in sterol biosynthesis mutants, and sterols notably share a common early-endocytic trafficking pathway with the PIN2 protein (Grebe et al. 2003, Willemsen et al. 2003). Moreover, the depletion of sterols from plant membranes leads to reduced endocytosis in plants (Kleine-Vehn et al. 2006).

During cytoskinesis, plants construct cell plates for the separation of a binucleated cell. PIN proteins are inserted into both sides of the plate, resulting in apical-basal localization following plate fusion with the plasma membrane. Sterols also seem to play a crucial role in the endocytosis-dependent reestablishment of apical PIN2 polarity after the division of epidermis cells (Men et al. 2008). Collectively, these results suggest that endocytic sterol trafficking and endocytosis or polar sorting events in plant cells are linked. It remains to be seen how sterols contribute to endocytosis in plants, but

sterols may define microdomains in plant membranes that regulate recruitment or retrieval from clathrin-coated pits.

Besides sterols, other lipids, such as phosphatidylinositol-related signals, are well established to affect vesicle trafficking in animal cells (McMaster 2001, Davletov et al. 2007). In contrast, only little is known on the role of these compounds in plant cells. One of the few reports shows that phospholipase D and its product, phosphatidic acid, appear to regulate the endocytosis rate and vesicle trafficking in general and the PIN2 protein in particular (Li & Xue 2007). In plant cells, as in animals, phosphatidylinositol-dependent signals may regulate endocytosis and vesicle trafficking.

Recent research has demonstrated the importance of endocytosis in a multitude of developmental and physiological processes in plants. Consequently, this field is finally receiving deserved attention and will rapidly progress in coming years.

Getting Back: Recycling in Plant Cells

Following the internalization of material from the plasma membrane, the regulation of the transfer of internalized receptors, transporters, or other molecules back to the plasma membrane is of tremendous importance for cell membrane integrity. In animal cells, various proteins are competent for recycling from distinct endosomal compartments to the plasma membrane. Furthermore, their endocytic routes are relatively well described by distinct molecular markers (Saraste & Goud 2007). In contrast, mechanisms and pathways that guide recycling in plants are still poorly characterized. Endocytic compartments in plants are often defined solely by their ability to incorporate lipophilic endocytic tracers (Bolte et al. 2004), making unambiguous designation of various early- and late-endocytic compartments difficult owing to differences in experimental conditions and possible compartment maturations.

The best-characterized cargo that exhibits constitutive recycling in plants is PIN1. The *Arabidopsis* BFA-sensitive endosomal ARF GEF GNOM, a vesicle transport regulator, is required for the polar localization and recycling of PIN1 (Steinmann et al. 1999, Geldner et al. 2001). Moreover, the utilization of an engineered BFA-resistant version of GNOM proved that the inhibitory effect of BFA on PIN1 cycling is due to the specific inhibition of GNOM (Geldner et al. 2003), indicating that GNOM defines the recycling rate of PIN1 to the plasma membrane. GNOM localizes to intracellular structures that are labeled by the endocytic tracer FM4-64 within 10 min and may define a recycling, but not an early, endosome (Geldner et al. 2003, Chow et al. 2008). GNOM does not exclusively mediate endosomal recycling of PIN proteins. Also, other, nonpolar plasma membrane cargos and cell wall components show BFA-sensitive, GNOM-dependent recycling and are affected in *gnom* loss-of-function mutants (Shevell et al. 2000, Geldner et al. 2003). Notably, the involvement of GNOM in basal-versus-apical targeting differs substantially. GNOM preferentially regulates recycling of PIN proteins to the basal plasma membrane, whereas apical localization of proteins at the apical plasma membrane is largely BFA insensitive and may be controlled by one or more BFA-resistant ARF GEFs (Kleine-Vehn et al. 2008a). Hence, apical and basal PIN targeting pathways are molecularly distinct by means of the ARF GEF vesicle trafficking regulators (Kleine-Vehn et al. 2008a), enabling simultaneous apical and basal polar PIN delivery in a single plant cell (**Figure 5**).

Basal cargos, such as PIN1, rapidly internalize in response to ARF GEF inhibition by BFA, implying that only recycling, but not internalization, of basal cargos is sensitive to BFA treatment (Geldner et al. 2001). This finding illustrates a possible employment of a BFA-resistant ARF GEF in cargo internalization from the basal plasma membrane. The GNL1, which is a BFA-resistant ARF GEF, may be involved in selective endocytosis of PIN proteins (Teh & Moore 2007). However, GNL1 is very important in ER-Golgi trafficking (Richter et al. 2007), and it remains to be seen whether GNL1

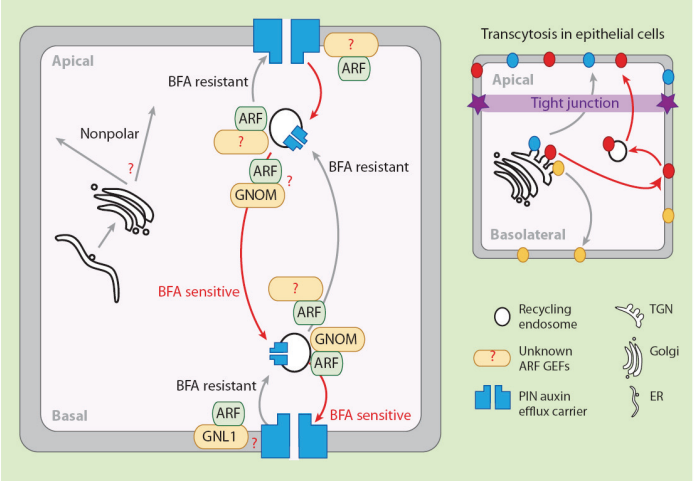

Figure 5

Transcytosis and apical and basal targeting of PIN-FORMED (PIN) proteins. Distinct ARF GEF–dependent apical and basal targeting pathways regulate polar PIN distribution. Alternative utilization of both pathways by the same PIN molecules enables dynamic translocation of PIN cargos between different cell sides. Inhibition of the GNOM component of the basal targeting pathway genetically or by BFA leads to the preferential recruitment of cargos by the apical pathway and to a reversible basal-to-apical PIN polarity shift. The top right panel illustrates that a similar process occurs in animal epithelial cells, in which several polar-competent proteins (depicted in *red*) are initially targeted to the basolateral cell side and subsequently transcytosed to their final destination (the apical cell side). However, other polar cargos (depicted in *yellow* and *blue*) do not require transcytosis for polar localization. Moreover, transcytosis in epithelial cells is also sensitive to BFA. Abbreviations used: ARF GEF, GDP/GTP exchange factor for adenosyl ribosylation factors; BFA, brefeldin A; ER, endoplasmic reticulum; GNL1, GNOM-LIKE1; TGN, trans-Golgi network.

directly or indirectly regulates the PIN endocytosis at the plasma membrane.

Besides the ARF GEF contribution in PIN recycling, SORTING NEXIN1 (SNX1) may define an endosome specific for PIN2, but not PIN1, trafficking (Jaillais et al. 2006) because PIN2 accumulates in SNX1 compartments that are distinct from the GNOM endosomes, after treatment with the phosphatidylinositol-3-kinase (PI3K) inhibitor wortmannin (Jaillais et al. 2006). However, pharmacological or genetic interference with the SNX1 compartment does not affect apical-basal polarity of PIN proteins but preferentially affects vacuolar sorting of plasma membrane proteins, including PIN2 (**Figure 6**) (Kleine-Vehn et al. 2008b). Enhanced PIN2 localization in the SNX1 compartment following gravity stimulation (Jaillais et al. 2006) coincides with enhanced vacuolar targeting and degradation of PIN2 (Abas et al. 2006, Kleine-Vehn et al. 2008b). Interestingly, SNX1 orthologs in yeast and animals are components of the retromer complex that assures the retrieval of vacuolar receptors back from the prevacuolar compartment (PVC) to the TGN (Seaman 2005). In plants, putative retromer components localize to the PVC and may interact with vacuolar sorting receptors (Oliviusson

Retromer: is important in recycling transmembrane receptors from PVCs/multivesicular bodies to the TGN

et al. 2006), which may also be a preferential role for SNX proteins in plants because SNX1 colocalizes with the putative plant retromer component VPS29 at the PVC (Oliviusson et al. 2006, Jaillais et al. 2008). VPS29 is required for storage vacuole formation during embryogenesis (Shimada et al. 2006), indicating that the putative plant retromer complex may be involved in general processes for the formation of multiple vacuole types (Bassham & Raikhel 2000). Furthermore, VPS29 is needed for endosome homeostasis, PIN protein cycling, and dynamic PIN1 repolarization during development (Jaillais et al. 2007). In one possible model, PIN1 first internalizes into GNOM-based endosomes and subsequently is recycled back via VPS29/SNX1-positive endosomes (Jaillais et al. 2007). However, inactivation of retromer-dependent receptor retrieval at the PVC may inhibit anterograde traffic from the PVC to the TGN. Because the TGN may act in plants as an early endosome (Dettmer et al. 2006), recycling of endocytosed cargo would be impaired indirectly (Jürgens & Geldner 2007). Alternatively, the retromer complex may have a gating function for endocytic vacuolar targeting of plasma membrane–localized proteins. Hence, the observed defects in *snx1* and *vps29* mutants (Jaillais et al. 2007, Kleine-Vehn et al. 2008b) may be explained by enhanced vacuolar targeting of PIN proteins (Kleine-Vehn et al. 2008b).

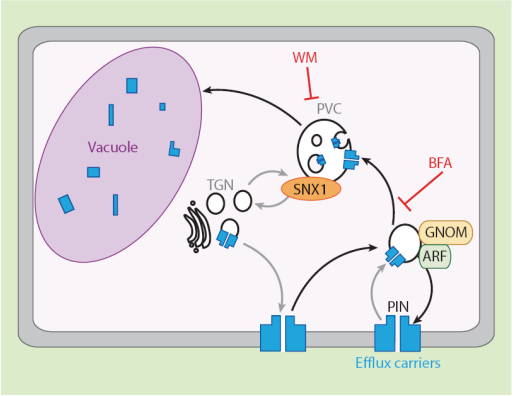

Figure 6

Vacuolar targeting of PIN-FORMED (PIN) proteins. Developmentally important posttranslational downregulation of PIN2 is realized by regulated targeting to the vacuole. PIN2 degradation is controlled by multiple sorting events at the plasma membrane, endosomes, and prevacuolar compartments (PVCs). The putative retromer complex component SORTING NEXIN1 (SNX1) is required for PVC identity. SNX1/VPS29-labeled PVCs appear to have a gating function for endocytic targeting to the vacuole. Other abbreviations used: ARF, adenosyl ribosylation factor; BFA, brefeldin A; TGN, trans-Golgi network; WM, wortmannin.

Going to the Other Side: Transcytosis Linking Endocytic Recycling and Polar Targeting

In animal epithelial cells, endocytic recycling is important for the establishment and maintenance of cell polarity (Rodriguez-Boulan et al. 2005, Leibfried & Bellaïche 2007). The endocytosis and subsequent retargeting to the other cell side by the process of transcytosis illustrate the tight linkage of endocytic recycling and polar targeting in animal cells (**Figure 5**).

In plants, apical and basal PIN targeting is realized by an alternative use of distinct polar targeting pathways by the same cargos (Kleine-Vehn et al. 2008a). Analysis of the GNOM contribution to apical-basal PIN targeting has yielded important mechanistic insights into dynamic polar PIN targeting. When GNOM-dependent, basal targeting is manipulated, for example, by pharmalogical or genetic inhibition of the GNOM function, basal PIN cargos internalize from the basal domain, first accumulate in the BFA compartments, and gradually appear at the apical cell side. This process is independent of de novo protein synthesis and, therefore, hints at a dynamic PIN translocation between distinct polar plasma membrane domains. Live-cell imaging with photoconvertible PIN2 versions visualizes the directional BFA-induced translocation from the basal plasma membrane, through endosomes, to the apical plasma membrane. After BFA removal, the basal localization of PINs is restored by a translocation in an

Prevacuolar compartment (PVC): a multivesicular body/membrane-bound organelle that sorts proteins from the Golgi apparatus to vacuoles, sending missorted proteins back to the Golgi and receiving endocytosed proteins from the plasma membrane

opposite direction from the apical-to-basal cell side (Kleine-Vehn et al. 2008a). Thus, PIN proteins move between the apical and basal sides of cells in a manner similar to that of the transcytosis mechanism known in animal cells, illustrating that endocytic recycling and polar targeting in plants are linked as well (**Figure 5**).

Sorting nexins have been implicated not only in receptor recycling at the TGN/PVC but also in transcytotic events in animal cells. In contrast, genetic or pharmacological interference with SNX1/VPS29-positive endocytic compartments does not seem to interfere directly with GNOM-dependent transcytosis in plant cells (Kleine-Vehn et al. 2008a). In agreement with these findings, SNX-dependent pathways seem to differ substantially between plants and animals. For instance, the human genome encodes for 47 PHOX domain proteins, of which approximately 30 are tentatively referred to as sorting nexins (Seet & Hong 2006). In contrast, *Arabidopsis* encodes only 11 PHOX domain–containing proteins (SMART search at **http://smart.embl-heidelberg.de**), of which 3 (AtSNX1, AtSNX2a, and AtSNX2b) show similarities to the human SNX1/SNX2 and 2 (AtSNX13a and AtSNX13b) show weak similarities to human SNX13. The small number of SNX-like proteins found in plants suggests low evolutionary divergence and, hence, a rather conserved SNX function in the putative plant retromer complex at the PVC/TGN interface.

In animal cells, a prominent example for transcytotic cargo is the transferrin receptor, which resides preferentially at the apical and/or basolateral plasma membranes in polarized epithelial cells. This receptor is able to transcytose from one plasma membrane domain to the other (Cerneus et al. 1993). Madin–Darby canine kidney cells predominantly display a basolateral plasma membrane localization of the transferrin receptor, which is subject to a basolateral-to-apical shift after BFA treatment (Wan et al. 1992, Shitara et al. 1998). The action of BFA on transferrin receptor transcytosis reflects the inhibitory effects of the drug on basolateral recycling, whereas transcytosis to the apical plasma membrane is unaffected (Wang et al. 2001). In an astonishing analogy, apical-to-basal transcytosis of PIN proteins displays a very similar involvement of BFA-sensitive ARF GEFs. Thus, basal targeting in polarized plant cells and basolateral localization in animal cells are remarkably analogous and may follow an evolutionarily conserved principle. However, in plants, the transcytosis mechanism may have acquired unique developmental roles because it may also regulate the dynamic changes in the PIN polarity that accompanies and mediates developmentally important processes such as tropisms and embryonic and postembryonic organ formation (Friml et al. 2002b, 2003b; Benkova et al. 2003; Kleine-Vehn et al. 2008a).

Despite obvious analogies between polar targeting mechanisms in plant and animal cells, the overall organization of the polar targeting machinery differs fundamentally. In animal epithelial cells, tight junctions function as barriers to the diffusion of some membrane proteins and lipids between apical and basolateral domains of the plasma membrane (Leibfried & Bellaïche 2007, Niessen 2007). In contrast, such a tight junction–like complex has not been observed in plant cells. Therefore, how plants facilitate the maintenance of distinct membrane compositions remains unclear. PIN proteins display only slow lateral diffusion within the plasma membrane (Dhonukshe et al. 2007a, Men et al. 2008). Thus, a constitutive transcytosis mechanism for polar PIN distribution may be rapid enough to counteract the lateral diffusion of PIN proteins within the plasma membrane and to constantly reestablish the apical-basal localization of cargos. Additionally, this mechanism can mediate the establishment of the polar localization of de novo–synthesized proteins. Polar targeting of de novo–synthesized PIN proteins seems to rely on a three-step mechanism that encompasses nonpolar PIN secretion, clathrin-dependent endocytosis, and subsequent polar endocytic recycling (Dhonukshe et al. 2008b). Therefore, it is tempting to speculate that a transcytosis mechanism regulates dynamic PIN polarity alterations during plant

development as well as establishes and maintains PIN polar localization in polarized cells.

Separating the Daughters: Endocytic Recycling in Cytokinetic Cell

Following mitosis, both animal and plant cells usually split a binucleated cell into two daughter cells. However, animal and plant cells evolved fundamentally different mechanisms of cytokinesis (Barr & Gruneberg 2007). By virtue of the rigid cell wall, plant cells, in contrast to the outside-in constriction of animal cells, construct a cell plate that is formed by intensive delivery and fusion of vesicles containing the components of the future plasma membrane and cell walls. Eventually, the growing cell plate fuses with the lateral sides of the cell, thus completing cytokinesis and separating the two daughter cells.

There is an ongoing debate concerning the origin of the cell plate–forming material. It remains unclear whether cell plate formation depends solely on the secretory pathway or whether endocytosis and, hence, endocytic recycling also contribute. Various endocytic tracers get rapidly incorporated from the extracellular space into the forming cell plate along with multiple plasma membrane proteins and cell wall material (Dhonukshe et al. 2006). Furthermore, endocytosis seems to be upregulated during cytokinesis, and the interference with endocytosis affects cell plate formation and cytokinesis (Dhonukshe et al. 2006). In contrast, the inhibition of secretion dramatically interferes with cytokinesis in plants, illustrating the importance of the secretion of the cytokinesis-specific syntaxin KNOLLE as well as other secreted molecules (Reichardt et al. 2007). In addition, pharmacological reduction of PI3K-dependent endocytosis does not lead to any obvious defects in cell plate formation (Reichardt et al. 2007).

Whether or to what degree endocytic recycling contributes to cell division in plants still remains to be seen. Established molecular tools to satisfactorily tackle this controversial issue in plants are lacking. Unraveling of the contribution of endocytic recycling or secretion is difficult because endocytosed and secreted materials are already merged in early-endosomal/TGN compartments (Dettmer et al. 2006; Lam et al. 2007a,b; Chow et al. 2008). Therefore, targeting of endocytosed material to the cell plate may constitute a default pathway because the endocytosed material may simply follow the bulked secretory flow from the TGN to the cell plate. In the most plausible scenario, which would be consistent with all the data, both the secretory and the endocytic components would contribute to cell plate formation. This model would allow simultaneous arrival of the secretory and endocytosed materials to the forming cell plate, not only to build it with de novo–synthesized material but also to identify it as a future cell surface by incorporating components specific to the mother cell's plasma membrane and cell wall (Dhonukshe et al. 2007b).

The scenario in which the cell plate also incorporates components of the cell surface presents a problem of polarity reestablishment of the polar cargos after completion of cell division. PIN proteins are also targeted to the forming cell plate (Geldner et al. 2001, Kleine-Vehn et al. 2008a); after the plasma membrane is formed, the PIN proteins would be present at both the apical and the basal sides of one of the daughter cells. To maintain the polarity of the mother cell in both daughter cells, there must be a mechanism whereby the polar cargos are stabilized on one side and retrieved from the opposite side of the newly formed cell wall. Very little is known as to which cellular and molecular mechanisms are involved. Sterols seem to play a crucial role in the reestablishment of apical PIN2 polarity (Men et al. 2008). The *cpi1* mutants are defective in endocytosis and deposit PIN2 at both the apical and the basal plasma membranes in post-cytokinetic cells (Men et al. 2008), suggesting a model in which sterol-dependent endocytosis retrieves PIN2 from the "wrong" side of the cell to reestablish uniform polarity in both daughter cells (**Figure 7**). It is possible that the internalized PIN2 is therefore resorted to the opposite,

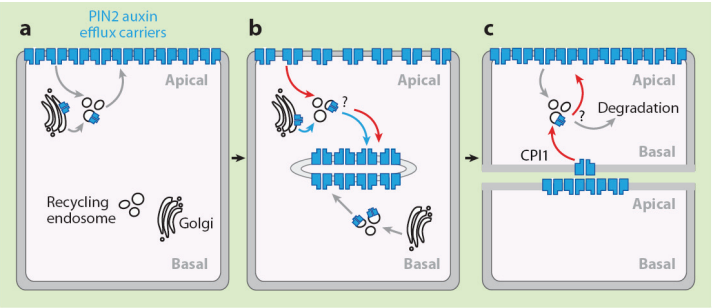

Figure 7

Sterol-dependent endocytosis of PIN2 in postcytokinetic cells. PIN2 displays preferential apical localization in interphase cells (*a*) but is deposited on both sides of the cell plate by transcytosis from the plasma membrane and/or by secretion (*b*). (*c*) PIN2 is retrieved from the newly formed basal plasma membrane by a sterol (CPI1)-dependent mechanism. Following internalization, the PIN2 proteins may translocate to the vacuole for degradation and/or may transcytose to the apical cell side.

"correct" side of the cell, which would be another demonstration of the role of transcytosis in plant cells. However, this scenario and whether similar mechanisms operate for other polar cargos need to be verified experimentally.

EXEMPLIFIED CASES: POLAR TARGETING AND ENDOCYTIC RECYCLING IN PLANT DEVELOPMENT

Induced Endocytosis in Plants

Eukaryotic cells have acquired an enormous adaptative capacity, enabling flexible responses to developmental or environmental cues. In animal cells, ligand- or substrate-induced endocytosis of plasma membrane–resident receptors or transporters has been studied extensively, suggesting a general mechanism for regulated endocytosis in response to external signals (Dugani & Klip 2005). In contrast, in plants, developmental and environmental cues that trigger differential endocytosis are poorly understood.

The first, and so far only, demonstration of ligand-induced receptor endocytosis was in the plant defense response against bacterial pathogens and concerned the bacterial peptide–based signal called flagellin. The *Arabidopsis* flagellin receptor FLS2 localizes to the plasma membrane in various cell types and, upon binding of the flagellin epitope, undergoes internalization in intracellular vesicles, likely leading to subsequent degradation of the receptor/ligand complex (Robatzek et al. 2006).

Interestingly, after binding of the ligand, FLS2 forms a complex with the brassinosteroid receptor–associated kinase BAK1, which seems to be crucial for its internalization (Chinchilla et al. 2007, Heese et al. 2007). Hence, the leucin-rich repeat receptor–like kinase BAK1 not only is instrumental for the brassinosteroid receptor BRI1 (Li et al. 2002, Russinova et al. 2004) but also plays a role in plant immunity by regulating ligand-induced endocytosis. However, although BAK1 may contribute to flagellin-induced FLS2 internalization, BRI1 internalization, recycling, and turnover are seemingly independent of brassinosteroid availability (Geldner et al. 2007). There are indications that brassinosteroids signal through BRI1 at the endosomal level, suggesting that the use of endosomes as signaling compartments is an unexpectedly broad phenomenon in eukaryotes (Geldner et al. 2007). In contrast, treatment

with another inhitor of vesicle trafficking, Endosidin1, leads to intracellular BRI1 accumulation and downregulates BRI1-dependent signaling, suggesting complex regulation of endosomal competence for potential brassinosteroid signaling (Robert et al. 2008). The utilization of a mutual coreceptor for two distinct receptors may influence the availability and/or kinetics of BAK1 binding and, hence, may be involved not only in brassinosteroid signaling or pathogen defense but also in cross talk of these two pathways.

Selective endocytosis has been demonstrated not only for plant receptors but also for some plasma membrane–resident transporters (Takano et al. 2005, Abas et al. 2006, Sutter et al. 2007). Prominent among such transporters is boron exporter BOR1 for xylem loading. Boron availability is crucial for plant development but toxic in high abundance. In the presence of high levels of boron, BOR1 is internalized into ARA7-positive endosomal compartments and is further targeted to the vacuole for degradation, suggesting a control mechanism for boron transporter presence at the cell surface by boron availability (Takano et al. 2005).

The potassium channel KAT1 accumulates in intracellular structures after abscisic acid concentrations are elevated (Sutter et al. 2007). Although the underlying mechanism is unknown, there may be an endocytosis-dependent mechanism for hormone-directed communication between the internal and external environments by the regulation of stomata opening and closure.

During some developmental processes, several PIN auxin efflux carriers also undergo substantial turnover and degradation in addition to constitutive endocytic recycling (Sieberer et al. 2000, Vieten et al. 2005, Abas et al. 2006, Kleine-Vehn et al. 2008b, Laxmi et al. 2008). As shown for PIN2 in **Figure 6**, following endocytosis, ARF GEF– and PI3K/SNX1-dependent sorting events at the endosomal and prevacuolar compartments contribute to the decision of whether to recycle or to translocate to the vacuole (Kleine-Vehn et al. 2008b). Auxin itself regulates PIN abundance at the plasma membrane by inhibiting PIN internalization from the plasma membrane (Paciorek et al. 2005). In addition, prolonged high auxin levels appear to induce PIN2 ubiquitination, internalization, and degradation (Vieten et al. 2005, Abas et al. 2006). Moreover, gravitropic stimulation triggers internalization (Abas et al. 2006) and vacuole-dependent degradation of PIN2 in epidermal cells at the upper side of the root (Kleine-Vehn et al. 2008b). This gravity-induced degradation of PIN2 occurs in cells with low, not high, auxin levels and may indicate posttranslational PIN2 downregulation in response to auxin depletion. It still needs to be seen whether boron, auxin, and possibly substrates for other plasma membrane–based transporters differentially downregulate their transporters by a conserved mechanism. Transient, transport-dependent conformational changes in carrier composition may enable conditional recruitment of machineries mediating, for instance, ubiquitination and subsequent internalization of their substrates.

Although the underlying pathways are largely unknown, the examples of ligand-induced receptor endocytosis, constitutive receptor cycling, and endosome-based signaling as well as the downregulation of receptors and transporters in response to substrate availability show that plant cells use all these endocytosis mechanisms to regulate their physiology. Undoubtedly, other examples of similar regulations will be identified in the coming years.

Integrating Developmental and Environmental Signals through Polarity Modulations

Intercellular auxin transport is the process in plants that makes most apparent the developmental output of subcellular dynamics and cell polarity (Berleth et al. 2007). Polar auxin transport is distinguished by its strictly controlled directionality, which is a crucial feature in auxin-mediated plant development (reviewed by Friml 2003, Zazímalová et al. 2007). The classical chemiosmotic hypothesis proposes that auxin flow polarity is determined by

the polar, subcellular localization of auxin efflux carriers (Rubery & Sheldrake 1974, Raven 1975; **Figure 1**). PIN proteins have been identified as one of the export carriers, and their polar subcellular localization indeed correlates with the direction of the auxin flow. The manipulation of PIN polarity has a clear impact on the ability of auxin to flow in a given direction, thus confirming that cellular PIN positioning is a determining factor in the directionality of polar auxin transport (Friml et al. 2004, Wisniewska et al. 2006, Boutté et al. 2007).

The finding that PIN proteins undergo permanent subcellular movements (Geldner et al. 2001, Dhonukshe et al. 2007a) was hard to reconcile with the original models of auxin transport. Hence, the important upcoming question concerns the functional role of this constitutive cycling. Besides exotic scenarios, such as neurotransmitter-like release of auxin from cells (Baluška et al. 2003), a plausible assumption is that constitutive trafficking provides the required flexibility for the rapid transcytosis-based PIN polarity changes, allowing rapid redirection of auxin flow in response to various signals, including environmental or developmental cues (Friml 2003). Indeed, rapid PIN relocations have been observed during embryonic development (**Figure 8**) (Friml et al. 2003b), aerial and underground organogenesis (**Figure 9**) (Benková et al. 2003, Reinhardt et al. 2003, Geldner et al. 2004, Heisler et al. 2005), vascular tissue formation (Scarpella et al. 2006), and root gravity responses (**Figure 10**) (Friml et al. 2002b). In all these instances, changes in PIN polarity are followed by the redirection of auxin fluxes and the rearrangement of local patterns of auxin accumulation (auxin gradients) that triggered the changes in the developmental programs (Kramer & Bennett 2006, Leyser 2006, Parry & Estelle 2006). An early PIN polarity switch signals root initiation during embryogenesis. At early stages of *Arabidopsis* embryo development, PIN7 is localized apically (toward the apical cell) in the suspensor, and PIN1 is mostly nonpolar in the proembryo, whereas at a later-defined stage, PIN1 polarizes to the basal side of cells adjacent to the future root meristem, and PIN7 changes its polarity from apical to basal (Friml et al. 2003b). These PIN polarity alterations lead to the rearrangement of auxin gradients and the accumulation of auxin at the presumptive embryo root pole and are among the necessary factors for root specification (Friml et al. 2003b, Weijers et al. 2005).

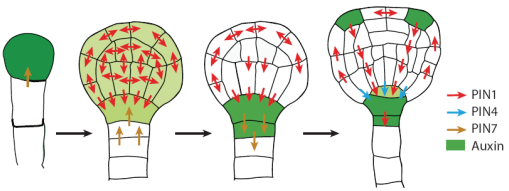

Figure 8

PIN-FORMED (PIN) polarity alterations during embryogenesis. Schematic representation of PIN distribution and polar orientation during *Arabidopsis* embryo development. PIN1 and PIN7 undergo a polarity switch at the globular stage. The GNOM-dependent focus of PIN1 to the basal sides of provascular cells coincides with an apical-to-basal shift of PIN7. These rearrangements of PIN polarity are accompanied by a dramatic change in the apical-basal auxin gradient. A new auxin maximum is established at the position of the future root, contributing to the initiation of the root specification. The analogy to GNOM-dependent transcytosis in polarized root cells may indicate polar transcytosis of PIN proteins during embryogenesis.

Another example of PIN polarity reorganization relates to the perception and response to environmental stimuli. Studies on *Arabidopsis* roots demonstrated that the PIN3 protein relocates in gravity-sensing cells of the root tip in response to gravistimulation (Friml et al. 2002b, Harrison & Masson 2008). When the root is reoriented into a horizontal position, gravity-sensing statoliths in the columella cells sediment, and PIN3 rapidly relocalizes from its originally uniform distribution to the new bottom side of these cells. The asymmetric repositioning of PIN3 is followed by redirection of the auxin flow downward, leading to auxin accumulation at the lower side of the root and, consequently, to downward root bending (**Figure 10**). It is possible that a similar mechanism involving PIN relocations underlies phototropic responses, but the connection between unidirectional light stimulus and PIN relocation has not been demonstrated. The

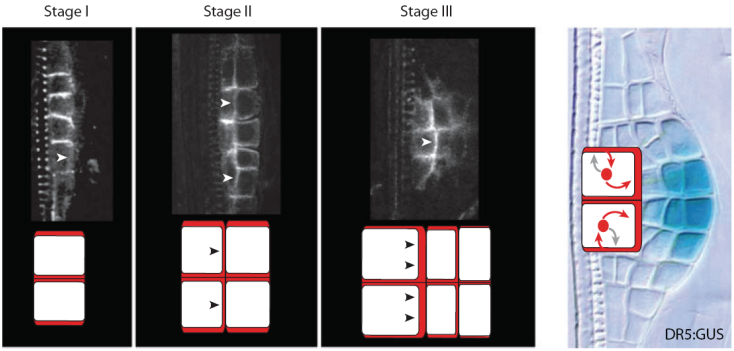

Figure 9

PIN-FORMED (PIN) polarity alterations during postembryonic organ formation. Immunolocalization and model of PIN1 localization during stage I to stage III (*first three panels*) of lateral root primordial development illustrate dynamic PIN1 polarity changes from the anticlinal toward the periclinal cell sides, pointing to the presumptive primordial tip. These changes coincide with the establishment of auxin maxima (visualized here by the DR5 auxin-responsive promoter activity; *fourth panel*) at the primordium tip. GNOM dependency of this event may reveal the involvement of dynamic PIN1 transcytosis between the anticlinal and the periclinal cell sides.

constitutive PIN subcellular dynamics may play a more direct role in the mechanism of auxin efflux because several potent and well-established inhibitors of auxin efflux act as stabilizers of the actin cytoskeleton and also inhibit PIN dynamics (Dhonukshe et al. 2008a). The precise connection between actin stabilization and mechanism of auxin efflux is, however, still unclear. Nonetheless, signal-induced rearrangements of PIN polarity in response to different inputs represent a plant-specific mechanism that integrates various internal and external signals and translates them into different developmental responses.

Canalization Hypothesis and the Effect of Auxin on Its Own Efflux

An important aspect linked to cell polarity and auxin transport relates to a rather fundamental issue in developmental biology: How does the individual cell in polarizing tissues know the polarity of its neighbors and the whole macroscopic context? In plant development, this issue has a pronounced importance because plants possess the remarkable ability to redefine cell and tissue polarities. Outstanding examples of auxin-dependent reorganization of plant tissues are the differentiation of vasculature during leaf venation, the connection of de novo–initiated organs with the preexisting vascular network, and vasculature regeneration after wounding. During these events, plant cells perceive their position within the tissue and can recognize their orientation relative to the rest of the plant body. Insights into underlying mechanisms are widely elusive, but efforts to tackle these processes have led to the formulation of the canalization hypothesis (Sachs 1981), whereby auxin can induce, by a positive-feedback mechanism, the capacity and polarity of its own transport, resulting in the gradual rearrangement of cell polarity and the repolarization of neighboring cells. Ultimately, new auxin conductive channels can be established, determining the position of new vascular strands. This intriguing hypothesis and other auxin-dependent self-organizing models (de Reuille et al. 2006, Jönsson et al. 2006, Smith et al. 2006, Kuhlemeier 2007, Merks et al. 2007)

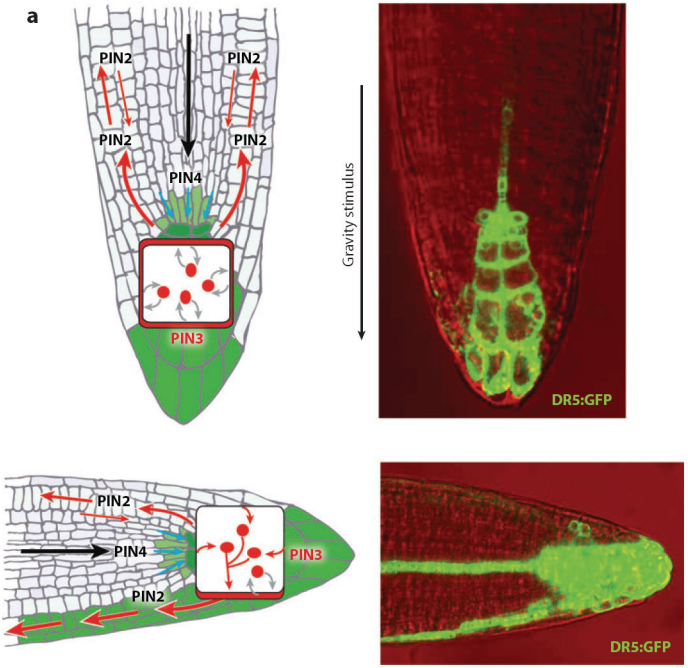

Figure 10

(*a*) PIN-FORMED (PIN)3 translocation during the root gravitropic response. Following gravity stimulation, PIN3 translocates rapidly to the bottom side of root cap cells and thus redirects auxin flow toward the lower side of the root. (*b*) PIN3- and PIN2-dependent asymmetric auxin distribution (visualized by the DR5:GFP-reliant auxin response) leads to differential growth and subsequent downward root bending. The dynamic nature of PIN3 targeting after gravity perception indicates a transcytosis-like mechanism for this polarity switch.

require the existence of positive-feedback regulation between auxin signaling and the capacity and polarity of auxin transport.

In fact, auxin feedback mechanisms regulating PIN activity, involving auxin-dependent regulation of transcription, degradation, and/or subcellular localization of auxin transport components, have been illustrated at multiple levels (Paciorek et al. 2005, Vieten et al. 2005, Sauer et al. 2006, Scarpella et al. 2006, Xu et al. 2006). On the transcriptional level, auxin-dependent cross-regulation of PIN expression may account for the extensive functional redundancy of PIN proteins, in which lack of function of one PIN protein leads to a transient increase in cellular auxin levels and transcriptional up-regulation of a functional ortholog (Vieten et al. 2005). Other auxin-dependent feedbacks have been identified at the level of PIN subcellular trafficking: Auxin interferes with endocytosis, including the internalization of PIN proteins, possibly by a mechanism independent of

auxin-induced transcription. This auxin effect leads to elevated PIN levels at the plasma membrane and increased auxin efflux (Paciorek et al. 2005). The underlying mechanism of the auxin effect is unclear but requires BIG, a callosin-like protein with an unclear function (Gil et al. 2001). By this mechanism, auxin regulates the PIN abundance and activity at the cell surface, accomplishing direct feedback regulation of auxin transport (Paciorek & Friml 2006).

Moreover, auxin indeed delegates the polarity of PIN proteins, hence influencing not only its own efflux rate but also its directional output (Sauer et al. 2006). This auxin effect is independent of PIN transcriptional regulation but involves the identified auxin/indole acetic acid (AUX/IAA) and auxin response factor–dependent signaling pathway (Parry & Estelle 2006, Kepinski 2007). Furthermore, auxin-dependent polarization cues are perceived in a cell type–dependent manner, eventually leading to averted polarity between neighboring cells (Sauer et al. 2006). These feedback regulations provide a conceptual framework for polarization during multiple regenerative and patterning processes in plants and are the unavoidable legacy of most models dealing with auxin-dependent patterning (Kramer 2008).

DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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Shows that subcellular polarity of the PIN localization is determined by the directionality of auxin flow during gravitropism.

Chapter 2

Result part I:

Subcellular Trafficking of the *Arabidopsis* Influx Carrier AUX1 Uses a Novel Pathway Distinct from PIN1

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Subcellular Trafficking of the *Arabidopsis* Auxin Influx Carrier AUX1 Uses a Novel Pathway Distinct from PIN1 [W]

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The directional flow of the plant hormone auxin mediates multiple developmental processes, including patterning and tropisms. Apical and basal plasma membrane localization of AUXIN-RESISTANT1 (AUX1) and PIN-FORMED1 (PIN1) auxin transport components underpins the directionality of intercellular auxin flow in *Arabidopsis thaliana* roots. Here, we examined the mechanism of polar trafficking of AUX1. Real-time live cell analysis along with subcellular markers revealed that AUX1 resides at the apical plasma membrane of protophloem cells and at highly dynamic subpopulations of Golgi apparatus and endosomes in all cell types. Plasma membrane and intracellular pools of AUX1 are interconnected by actin-dependent constitutive trafficking, which is not sensitive to the vesicle trafficking inhibitor brefeldin A. AUX1 subcellular dynamics are not influenced by the auxin influx inhibitor NOA but are blocked by the auxin efflux inhibitors TIBA and PBA. Furthermore, auxin transport inhibitors and interference with the sterol composition of membranes disrupt polar AUX1 distribution at the plasma membrane. Compared with PIN1 trafficking, AUX1 dynamics display different sensitivities to trafficking inhibitors and are independent of the endosomal trafficking regulator ARF GEF GNOM. Hence, AUX1 uses a novel trafficking pathway in plants that is distinct from PIN trafficking, providing an additional mechanism for the fine regulation of auxin transport.

INTRODUCTION

The signaling molecule auxin mediates a surprising variety of plant developmental events, including embryo, root, and vascular tissue patterning, organ and fruit development, tropisms, apical dominance, and tissue regeneration (reviewed in Tanaka et al., 2006). During processes such as tissue regeneration or de novo organ formation, plants rearrange and respecify the polarity of fully specified cells. The connection between cellular polarizing events and the macroscopic manifestation of polarity, such as the specification of different cell types along the axis, largely depends on the directional (polar) transport of auxin (Friml et al., 2003).

Auxin moves actively in a strictly controlled direction from the shoot apex toward the root base by the action of a specialized transport system (reviewed in Benjamins et al., 2005) composed of specific influx and efflux carriers, which mediate auxin flow into and out of cells, respectively. It has been hypothesized that the polarity of auxin flow results from differences at the single cell level between apical and basal membranes in their relative permeabilities to auxin (Rubery and Sheldrake, 1974; Raven, 1975). Candidate genes coding for the critical components of auxin influx and efflux carriers have been identified by molecular genetic studies in *Arabidopsis thaliana*. The components of the auxin efflux complexes of the PIN-FORMED (PIN) family are plasma membrane (PM)–localized, plant-specific proteins that are able to facilitate the efflux of auxin from cells (Petrasek et al., 2006). Importantly, PIN proteins are distributed asymmetrically in auxin transport-competent cells (Gälweiler et al., 1998; Müller et al., 1998; Friml et al., 2002a, 2002b, 2003; Benková et al., 2003), and this polar localization determines the direction of auxin flow at least in meristematic tissues (Wiśniewska et al., 2006).

Physiological experiments indicated that efflux carrier proteins have a very short half-life at the PM (reviewed in Morris, 2000). Molecular–genetic studies confirmed this notion and identified a regulator of subcellular trafficking—GNOM—as a critical component of the delivery of PIN proteins from the endosomes to the PM (Geldner et al., 2001, 2003). *GNOM* (also called *EMB30*) encodes an endosomal, brefeldin A (BFA)–sensitive ARF GEF (for ADP-ribosylation factor GDP/GTP exchange factor) (Shevell et al., 1994), whose inhibition by BFA leads to the disappearance of PINs from the PM and their intracellular accumulation in so-called BFA compartments (Steinmann et al., 1999). This effect is fully reversible and reveals a constitutive cycling of PINs between PM and endosomes (Geldner et al., 2001). Although a biological role for the cycling of PIN proteins is not clarified yet, it may serve as a mechanism for rapid changes in PIN polarity in response to environmental (Friml et al., 2002a) or developmental (Benková et al., 2003; Friml et al., 2003; Reinhardt et al., 2003) cues and as a means of feedback regulation of auxin transport by subcellular PIN translocation (Paciorek et al., 2005).

The auxin influx carrier protein AUXIN-RESISTANT1 (AUX1) belongs to the amino acid permease family of proton-driven transporters and therefore was favored to play a role in the uptake of the Trp-like auxin molecule indole-3-acetic acid (Bennett et al., 1996). The unique features of the phenotype conferred by the *aux1* mutant, which can be rescued specifically

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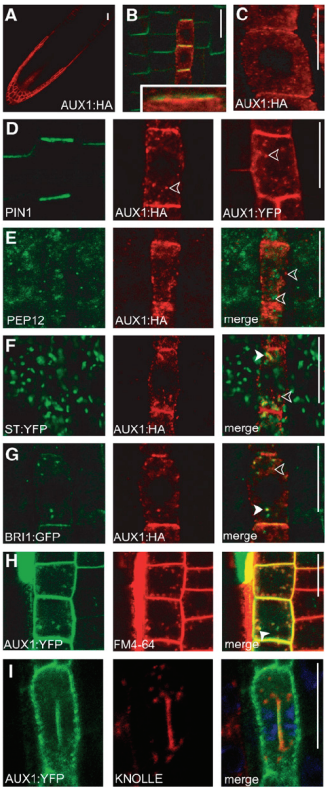

Figure 1. PM and Intracellular Accumulation of AUX1.

(A) to **(C)** Localization of AUX1 (red) and PIN1 (green) auxin transport components.
(A) AUX1 localization pattern in the root tip.
(B) Apical localization of AUX1:HA and basal localization of PIN1 in protophloem cells.
(C) Axial (apical and basal) localization of AUX1:HA in epidermis cells.
(D) AUX1:HA and AUX1:YFP in protophloem cells (red) but not PIN1 (green) show pronounced accumulation in intracellular compartments (indicated by arrowheads).
(E) No colocalization of AUX1:HA (red) and prevacuolar compartment PEP12 (green) in protophloem cells.
(F) Partial colocalization of AUX1:HA (red) and the Golgi apparatus marker ST:YFP (green) in protophloem cells.
(G) Partial colocalization of AUX1:HA (red) and endosomal BRI1:GFP (green) in protophloem cells.
(H) Partial colocalization of AUX1:YFP (green) and the endocytic tracer FM4-64 (red) in epidermis cells.
(I) During cell division, AUX1:HA (green) colocalizes at the forming cell plate with KNOLLE (red). 4′,6-Diamidino-2-phenylindole–stained nuclei in protophloem cells are shown in blue.
Immunocytochemistry is shown in **(A)** to **(G)** and **(I)**, and live-cell imaging is shown in **(H)**. Closed arrowheads show colocalizing compartments, and open arrowheads show noncolocalizing compartments (**[E]** to **[H]**). Bars = 10 μm.

by membrane-permeable auxins, and auxin uptake activity in heterologous systems strongly support the role of AUX1 as an auxin influx carrier (Yamamoto and Yamamoto, 1998; Marchant et al., 1999; Yang et al., 2006). An epitope-tagging approach showed that the AUX1 protein is expressed in protophloem, columella, lateral root cap, and epidermal cells in the *Arabidopsis* root apex (Swarup et al., 2001). Interestingly, in protophloem cells, AUX1 shows a pronounced polar localization at the apical (upper) side of cells (Swarup et al., 2001) opposite to basally (lower side) localized PIN-FORMED1 (PIN1) in the same cells (Friml et al., 2002b). Like PIN proteins, AUX1 localization seems to exhibit BFA sensitivity (Grebe et al., 2002), and AUX1, but not PIN1, trafficking is dependent on the novel endoplasmic reticulum protein, AUXIN-RESISTANT4 (AXR4) (Dharmasiri et al., 2006).

The aim of this study was to characterize AUX1 trafficking and determine its subcellular targeting pathway. Using the unique situation in the protophloem, where AUX1 and PIN1 are polarly localized at the opposite sides of the same cell, the mechanisms of AUX1 and PIN trafficking can be compared. Such data should lead toward a better understanding of the cell biological determinants governing polar auxin transport and also extend our knowledge regarding the apical and basal polar trafficking pathways in plant cells.

RESULTS

AUX1 and PIN1 Localize to the Opposite Sides of Protophloem Cells and Target to the Forming Cell Plate

We analyzed AUX1 subcellular distribution using hemagglutinin (HA)- and yellow fluorescent protein (YFP)–tagged proteins (Swarup et al., 2001, 2004). As shown previously, AUX1 is expressed in epidermis, lateral root cap, columella, and protophloem cells of the root tip (Figure 1A). AUX1 signal can often be found at all cell sides but is regularly enriched at the apical (upper) PM of protophloem cells (Figures 1B and 1D), at both the apical and basal (lower) sides of epidermis cells (Figure 1C), and without pronounced asymmetric distribution in other cell types such as the root cap (see Figure 5J below). By contrast, PIN1 is localized on the basal side of the root stele cells (Friml et al., 2002b), including protophloem (Figure 1B). Importantly, in protophloem cells, AUX1 and PIN1 show localization at opposite sides of the same cell (Figure 1B, inset). PIN proteins (Geldner et al., 2001) along with many other PM proteins (Dhonukshe et al., 2006) have been shown to accumulate in the developing cell plate in dividing cells. Interestingly, AUX1, like PIN1, was detected at cell plates of

dividing protophloem cells, where it colocalized with the cytokinesis-specific syntaxin KNOLLE (Figure 1I).

The occurrence of AUX1 and PIN1 at the opposite sides of protophloem cells suggests that AUX1 and PIN1 are targeted by divergent vesicle trafficking pathways. Furthermore, it indicates that both apical and basal targeting/retrieval operates at the end of cell plate formation, thus establishing PIN1 and AUX1 at opposite sides of the completed cell wall.

AUX1 Also Localizes to the Golgi Apparatus and Endosomes

In addition to their polar PM localization, the protophloem-expressed AUX1:HA and AUX1:YFP fusion proteins exhibit a pronounced intracellular signal (Figure 1D). To characterize the identity of AUX1-labeled endomembranes, we used several established subcellular markers.

No colocalization (Figure 1E; see Supplemental Figure 2 online) was observed with a prevacuolar compartment marker in *Arabidopsis* SYNTAXIN21 (SYP21/PEP12) (da Silva Conceição et al., 1997). By contrast, Golgi apparatus markers γ-COAT PROTEIN (γ-COP) (Ritzenthaler et al., 2002; Geldner et al., 2003) (data not shown) and ST:YFP (Grebe et al., 2003) (Figure 1F; see Supplemental Figure 2 online) exhibited partial colocalization with AUX1:HA signal, suggesting that a percentage of the intracellular AUX1 protein resides at the Golgi apparatus. AUX1 localization at the Golgi apparatus was often variable, suggesting a complex regulation of AUX1 biosynthesis. As PIN proteins are known to recycle between PM and endosomes, we tested whether a proportion of intracellular AUX1 also resides on endosomes. The *Arabidopsis* brassinosteroid receptor BRASSINOSTEROID-INSENSITIVE1 (BRI1) has been reported to reside at the PM and endosomes (Russinova et al., 2004). Simultaneous visualization of BRI1:green fluorescent protein (GFP) and AUX1:HA revealed partial colocalization of intracellular signals (Figure 1G; see Supplemental Figure 2 online).

In other cell types, including epidermal cells, in which AUX1 function has been linked to trichoblast polarity events (Grebe et al., 2002), the intracellular pool of AUX1 was also detected. Real-time, live-cell imaging showed that AUX1:YFP-positive structures are highly dynamic and colocalize with the endocytic tracer lipophilic dye FM4-64 at early stages (<5 min) of its internalization (Figure 1H; see Supplemental Movie 1 online). This further confirms that part of intracellular AUX1 resides in endosomes. Collectively, these data show that the intracellular pool of AUX1 resides on both dynamic endosomal and Golgi apparatus membranes.

Intracellular but Not PM AUX1 Localization Is BFA Sensitive

In *Arabidopsis*, BFA blocks trafficking from recycling endosomes to the PM and causes endosomes and internalized endocytic cargo to aggregate into so-called BFA compartments, which become surrounded by Golgi stacks (Geldner et al., 2003). It has been shown previously that both PIN1 and AUX1 accumulate in BFA compartments (Geldner et al., 2001; Grebe et al., 2002).

PIN1 and AUX1 aggregation in BFA compartments is fully reversible, as removal of BFA in the presence of the protein synthesis inhibitor cycloheximide (50 μM, 1 h) leads to the reestablishment of PIN1 (Geldner et al., 2001) and AUX1 localization at the PM and subcellular structures (see Figure 4A below). In addition, latrunculin B (Lat B)–dependent depolymerization of the actin cytoskeleton inhibits PIN1 and AUX1 accumulation in BFA compartments (Geldner et al., 2001) (data not shown), confirming the BFA-sensitive, actin-dependent distribution of PIN1 and AUX1 proteins.

Next, we compared the dynamics of PIN1 and AUX1 translocation in response to BFA. PIN1 was almost completely internalized within 2 h (i.e., the signal disappeared from the basal PM and appeared in BFA compartments) (Figure 2A). Under similar conditions (50 μM BFA, 2 h), AUX1 showed weak intracellular agglomeration, with the majority of the AUX1 signal remaining at the PM (Figure 2A). Even at higher BFA concentrations (up to 200 μM) and extended treatment periods (up to 6 h), the AUX1 signal strongly persisted in the PM (data not shown). Moreover, PM localization of AUX1 in epidermis cells also persisted after prolonged BFA treatment (see Figures 4A and 4B below), suggesting a similar, BFA-resistant targeting mechanism to the PM in both cell types.

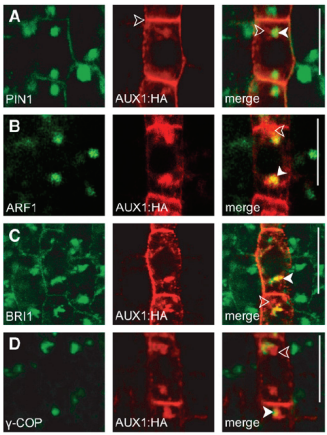

Figure 2. BFA-Sensitive Dynamics of AUX1 in Protophloem Cells.

(A) Partial colocalization of PIN1 (green) and AUX1:HA (red) in BFA compartments.
(B) Partial colocalization of endosomal ARF1 (green) and AUX1:HA (red) in BFA compartments.
(C) Partial colocalization of endosomal BRI1:GFP (green) and AUX1:HA (red) in BFA compartments.
(D) Partial colocalization of the Golgi marker γ-COP (green) and AUX1:HA (red) in BFA compartments.
Closed arrowheads show colocalizing compartments, and open arrowheads show noncolocalizing compartments. Immunocytochemistry imaging is shown in all panels. Bars = 10 μm.

Furthermore, although the typical PIN1 signal in BFA compartments was uniformly rounded, the AUX1 accumulation in protophloem cells was more dispersed and irregularly shaped. Colocalizations after BFA treatment (50 μM BFA, 2 h) showed that PIN1 partially colocalized with AUX1, often in the center of the BFA compartments (Figure 2A; see Supplemental Figure 2 online). Here also, the endosomal markers BRI1 and ADP-RIBOSYLATION FACTOR1 (ARF1) (Xu and Scheres, 2005) showed pronounced colocalization with internalized PIN1 (Paciorek et al., 2005), whereas only partial colocalization was observed with AUX1 in BFA compartments (Figures 2B and 2C; see Supplemental Figures 2 and 3 online). On the other hand, the Golgi apparatus markers γ-COP and ST:YFP did not colocalize with PIN1 in BFA compartments (Geldner et al., 2003) but showed partial colocalization with AUX1 (Figure 2D; data not shown; see Supplemental Figure 2 online). In accordance with the PEP12/AUX1 colocalization study, PEP12 did not colocalize with BRI1 in BFA compartments (see Supplemental Figure 1 online).

These findings confirm that AUX1 accumulates in BFA compartments. However, this relates to the aggregation of AUX1-containing endosomal and Golgi apparatus–derived structures. In contrast with PIN1, the prolonged BFA treatments did not visibly affect AUX1 localization at the PM, suggesting a BFA-resistant mechanism of AUX1 targeting to the PM.

Fluorescence Recovery after Photobleaching Analysis Reveals a Connection between Intracellular and PM Pools of AUX1

Unlike in the case of PIN1 (Geldner et al., 2001), the BFA treatment experiments did not unambiguously demonstrate a constitutive endosomal recycling of AUX1 to and from the PM.

To examine whether there is any exchange of AUX1 between the PM and the intracellular pool, we performed fluorescence recovery after photobleaching (FRAP) experiments. In protophloem and epidermis cells, the PM pool of AUX1:YFP recovered visibly within 20 min after bleaching (Figures 3A and 3D). Because TIBA (50 μM, 1 h), which inhibits actin-dependent protein recycling (Geldner et al., 2001), impeded AUX1 recovery at the PM (Figure 3F; see Supplemental Figure 4 online), we concluded that AUX1 recovery is conditioned by trafficking-dependent delivery rather than by lateral diffusion. This is in accordance with the constant arrival of AUX1-containing intracellular structures at the PM, as visualized by live-cell imaging (see Supplemental Movie 1 online). When protein synthesis was inhibited by cycloheximide (50 μM, 0.5 h) (Geldner et al., 2001), the recovery of AUX1 at the PM was slower but nevertheless occurred within 30 min (Figure 3B). This finding shows that the PM pool can be replenished by AUX1 from the preexisting intracellular pool.

When the PM pool of AUX1:YFP in protophloem cells was bleached and the cells were cotreated with BFA, AUX1 still accumulated in BFA compartments (Figure 3C), confirming only an insignificant contribution of PM-localized AUX1 to BFA compartments. Importantly, in this case (Figure 3C) and also when cells were pretreated with BFA (50 μM, 1 h) (Figure 3E), we still observed AUX1 recovery at the PM after 20 to 30 min in both protophloem and epidermis cells.

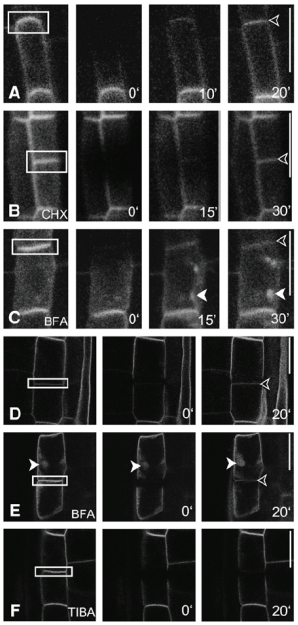

Figure 3. FRAP Analysis of AUX1 Dynamics.

FRAP analysis of AUX1:YFP in protophloem (**[A]** to **[C]**) and epidermal cells (**[D]** to **[F]**).
(A) AUX1:YFP incidence at the apical PM of protophloem cells recovers within 20 min after photobleaching.
(B) AUX1:YFP recovery at the apical PM is delayed (30 min) when protein synthesis is inhibited by cycloheximide (CHX).
(C) AUX1:YFP label also accumulates in BFA compartments when the PM pool of AUX1 is photobleached. The recovery at the PM also occurs to some extent in the presence of BFA.
(D) AUX1:YFP recovery in untreated epidermal root cells after 20 min.
(E) AUX1:YFP recovery at the PM after BFA compartment formation occurs within 20 min.
(F) TIBA interferes with AUX1:YFP recovery at the PM.
Open arrowheads depict recovered AUX1 at the PM, and closed arrowheads depict recovered AUX1 at the BFA compartments. Bars = 10 μm.

Hence, the intracellular and PM pools of AUX1 are interconnected, but AUX1 protein delivery to the PM is BFA-insensitive and AUX1 occurrence in the BFA compartments is to a large extent attributable to the fusion of the intracellular Golgi apparatus and endosomal membranes containing AUX1 protein. Thus, in contrast with PIN1, which needs the action of BFA-sensitive ARF GEF GNOM, constitutive AUX1 delivery to the PM likely involves BFA-resistant ARF GEFs.

Subcellular Trafficking of AUX1 Is GNOM Independent

Although the delivery of AUX1 to the PM seems to involve BFA-resistant ARF GEFs, AUX1 subcellular dynamics also require the activity of BFA-sensitive ARF GEFs (Grebe et al., 2002), as the intracellular AUX1 pool reversibly accumulates in BFA compartments (Figure 4A). We addressed whether this process involves the BFA-sensitive ARF GEF GNOM, which is a major ARF GEF involved in the trafficking of PIN proteins (Geldner et al., 2003). AUX1:YFP was coexpressed in a transgenic line in which the wild-type GNOM protein was replaced by the BFA-resistant GNOM (GN) version (GN^{M696L}-myc) (Geldner et al., 2003). The delivery of PIN1 to the PM is to a large extent BFA-resistant in this line, as BFA treatment does not lead to the accumulation of PIN1 in the BFA compartments (Geldner et al., 2003). By contrast, AUX1:YFP still readily accumulated in BFA compartments after BFA (50 μM, 2 h) treatment in GN^{M696L}-myc transgenic lines in both epidermis and protophloem cells (Figure 4B, inset). Moreover, the protein amount and time dependence was fully comparable to AUX1 accumulation in the wild-type control. This demonstrates that the BFA-induced agglomeration of AUX1, unlike PIN1, is independent of GNOM function and requires the action of other BFA-sensitive ARF GEFs. This observation further emphasizes the differences in the mechanisms of PIN1 and AUX1 trafficking.

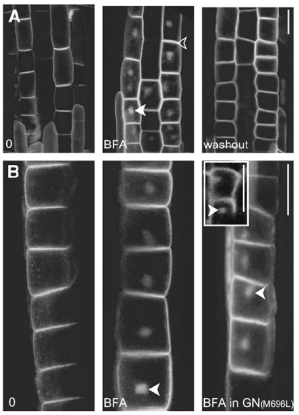

Figure 4. GNOM-Independent Dynamics of AUX1 in Epidermal Cells.

(A) AUX1:YFP shows reversible accumulation in BFA compartments after BFA treatment.

(B) AUX1:YFP also readily accumulates in BFA compartments in the BFA-resistant GNOM line. The inset shows the BFA-dependent accumulation of AUX1:YFP in protophloem cells.

The open arrowhead indicates that AUX1 remains constant at the PM, and closed arrowheads indicate BFA compartments. Live-cell imaging is shown in all panels. Bars = 10 μm.

AUX1 Localization and Intracellular Dynamics Require the Actin Cytoskeleton

Next, we analyzed the cytoskeletal requirements for the guidance of AUX1-containing vesicles. We interfered with the cytoskeleton using the microtubule-depolymerizing agent oryzalin (Hugdahl and Morejohn, 1993) and the actin depolymerizer Lat B (Spector et al., 1983). A similar experimental strategy previously revealed that PIN protein dynamics primarily require actin filaments but not microtubules (Geldner et al., 2001; Friml et al., 2002a).

Oryzalin treatments (20 μM, 3 h) were sufficient to disrupt microtubules (Geldner et al., 2001) but did not visibly interfere with AUX1 trafficking. Only treatments with high oryzalin concentrations (40 μM, 3 h), which lead to changes in cell morphology and therefore might induce secondary effects, affected AUX1 polar localization (see Supplemental Figure 5 online). These results suggest that microtubules are not primarily required for AUX1 polar localization and subcellular dynamics.

By contrast, actin depolymerization by Lat B (30 μM, 3 h) led to intracellular agglomeration of the AUX1 signal in protophloem (Figure 5B) or epidermis (data not shown) cells. Lat B treatments also affected the polarity of PIN1 and AUX1 localization to some extent, as treated seedlings showed more randomly distributed PIN1 and AUX1 signals in protophloem cells (Figure 5B). Furthermore, analysis of the concentration dependence of the Lat B effect showed that a lower Lat B concentration (20 μM, 3 h), which did not affect PIN1 localization, was sufficient to strongly influence AUX1 targeting (Figure 5A).

In summary, these data show that AUX1 trafficking and polar PM localization are not strictly dependent on microtubules but require intact actin filaments. Moreover, AUX1 localization at the PM is more strictly dependent on the actin cytoskeleton than is PIN1 localization.

Effects of Auxin Transport Inhibitors on AUX1 Dynamics

Auxin has been shown to inhibit the endocytic step of PIN cycling (Paciorek et al., 2005). In addition, auxin efflux inhibitors such as TIBA and PBA interfere with the intracellular cycling of PIN proteins (Geldner et al., 2001). Recently, another class of inhibitors, such as 1-NOA, which preferentially targets auxin influx, has been identified, but the molecular mechanism of their action remains unknown (Parry et al., 2001). However, all of these inhibitors interfere with auxin transport–dependent auxin distribution within tissues, as monitored by DR5-based auxin response reporters (Sabatini et al., 1999; Ottenschläger et al., 2003).

We tested the effects of auxin efflux and influx inhibitors on AUX1 trafficking. It appeared that efflux-dependent auxin flow is necessary for the polar PM localization of AUX1 and PIN1, because treatments with the auxin efflux inhibitor naphthylphthalamic acid (50 μM, 3 h) largely abolished the polar localization of both proteins (Figure 5D). On the other hand, inhibition of influx by 1-NOA treatment, even at high concentrations (100 μM, 3 h), did not interfere substantially with polar PM or intracellular localization of AUX1 or PIN1 (Figure 5E). Moreover, both PIN1 and AUX1 readily accumulated in BFA compartments in the

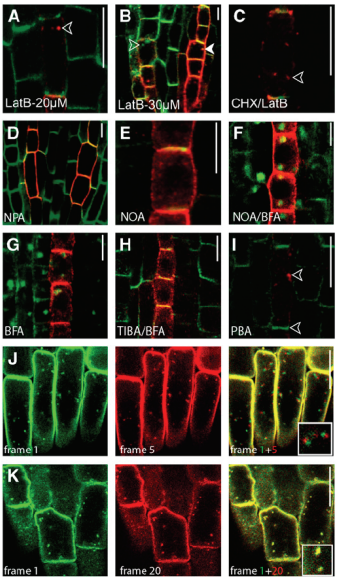

Figure 5. Auxin and Auxin Transport Inhibitors: Effects on AUX1 Dynamics.

(A) The actin-depolymerizing agent Lat B preferentially targets AUX1 and causes AUX1 retrieval from the PM and its aggregation.
(B) At higher Lat B concentrations, PIN1 is also affected.
(C) AUX1 aggregation also occurs when protein synthesis is inhibited. CHX, cycloheximide.
(D) Naphthylphthalamic acid (NPA) affects the polar distribution of AUX1 and PIN1.
(E) 1-NOA does not affect the localization of AUX1 and PIN1.
(F) AUX1 and PIN1 readily accumulate in BFA compartments in the presence of 1-NOA.
(G) BFA treatment leads to AUX1/PIN1 accumulation in BFA compartments.
(H) TIBA inhibits the BFA-induced aggregation of AUX1/PIN1.
(I) PBA treatment causes the internalization and subcellular aggregation of AUX1 but not of PIN1 (indicated by the lower arrowhead).
(J) Subcellular dynamics of AUX1:YFP (yellow). Overlay of frame 1 (green) on frame 5 (+30 s; red).
(K) TIBA blocks the subcellular dynamics of AUX1:YFP. Overlay of frame 1 (green) on frame 20 (+120 s; red).
Open arrowheads indicate AUX1 accumulation, and the closed arrowhead shows AUX1/PIN1 colocalization. Immunocytochemistry of protophloem cells is shown in **(A)** to **(I)**, and live-cell imaging of lateral root cap cells is shown in **(J)** and **(K)**. The localization of AUX1:HA is shown in red, and that of PIN1 is shown in green in **(A)** to **(I)**. Bars = 10 μm.

presence of 1-NOA in protophloem (Figure 5F) and epidermal (see Supplemental Figure 6A online) cells. These results suggest that the 1-NOA inhibition of auxin influx is not attributable to an effect on AUX1 polarity or trafficking.

By contrast, TIBA and PBA have profound effects on AUX1 trafficking. TIBA (50 μM, 30-min pretreatment) inhibited the BFA-induced (50 μM, 2 h) aggregation of AUX1 in protophloem (Figures 5G and 5H) and epidermis (see Supplemental Figure 6B online). Furthermore, the recovery of AUX1 from the BFA compartments was completely inhibited when BFA was washed out with TIBA or PBA (see Supplemental Figure 6C online). These results suggest that both TIBA and PBA inhibit trafficking not only of PIN1 (Geldner et al., 2001) but also of AUX1. To test directly the effect of these drugs on AUX1 trafficking, we performed live-cell imaging experiments in the absence and presence of these drugs. In the lateral root cap cells, AUX1:YFP-labeled membranes displayed highly dynamic behavior (see Supplemental Movie 1 online), which was visualized by a superimposition of successive frames 1 and 5 (6-s interval between frames, color-coded green and red; Figure 5J). Strikingly, in the presence of TIBA (50 μM) or PBA (10 μM), AUX1 motility was blocked (see Supplemental Movie 2 online and the superimposition of frames 1 and 20 in Figure 5K). By contrast, treatment with auxins (50 μM naphthylacetic acid) did not influence AUX1 dynamics (see Supplemental Movie 3 and Supplemental Figures 6E to 6G online). Inhibition of AUX1 dynamics for a prolonged period (PBA, 5 μM, 3 h; TIBA, 50 μM, 5 h) led to subcellular aggregation of AUX1 in the protophloem (Figure 5I) and epidermis (data not shown). These effects seem to be more specific for AUX1 trafficking, because PIN1 localization is affected to a much lesser extent and only at higher concentrations or longer treatments.

These data collectively demonstrate that TIBA and PBA, but not 1-NOA or auxins, block the subcellular trafficking of the AUX1 protein. Moreover, AUX1 trafficking is more sensitive than PIN1 to PBA/TIBA action, which may reflect stricter requirements for AUX1 trafficking along actin filaments and/or actin-dependent protein anchoring of AUX1 at the PM.

Sterol Composition of Membranes Affects AUX1 Targeting

Sterols are known to be important for polar sorting events in animal cells (Keller and Simons, 1998; Michaux et al., 2000). Recent studies suggested sterol involvement in polar protein targeting in plant cells, as PIN1 polarity was perturbed in the *orc* mutant, which is deficient in STEROL METHYLTRANSFERASE1 (SMT1) function (Willemsen et al., 2003). In animals, filipin specifically binds sterols (Miller, 1984), and high filipin doses are known to shift or deplete sterols in membranes of animal cells, leading to the inhibition of endocytosis (Marella et al., 2002). In plant cells, filipin also binds sterols (Grebe et al., 2003) and inhibits FM4-64 uptake as well as the endocytosis of PM-resident proteins (Figures 6A to 6E), suggesting a comparable mode of filipin action in animal and plant cells.

Filipin treatment (100 μM, 1.5 h) leads to strong AUX1 accumulation in aggregates preferentially associated with the lateral PM in protophloem (Figure 6F) but not in epidermis or lateral root cap cells (see Supplemental Figure 7 online). In the case of PIN1, filipin interfered slightly with polar localization, but no lateral

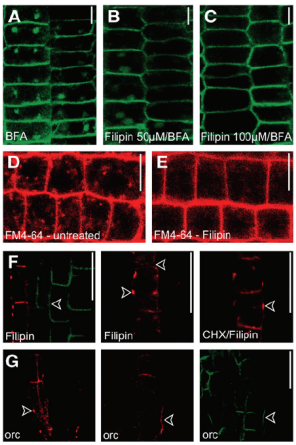

Figure 6. Sterol Requirements of AUX1 and PIN1 Dynamics.

(A) Aquaporin PIP2:GFP-expressing epidermal root cells show accumulation in BFA compartments.
(B) Pretreatment with 50 μM filipin shows weak effects on BFA-induced internalization of PIP2.
(C) Filipin treatment (100 μM) prevents PIP2 accumulation in BFA compartments.
(D) FM4-64 uptake (35 min) in untreated epidermal root cells.
(E) FM4-64 uptake (35 min) in filipin-treated (100 μM) epidermal root cells.
(F) The sterol binding agent filipin preferentially targets AUX1 and leads to AUX1 aggregation predominantly at the lateral cell sides. The polarity of PIN1 localization also is affected. AUX1 aggregation also occurs when protein synthesis is inhibited. CHX, cycloheximide.
(G) Genetic interference with sterol composition in *orc* mutants leads to AUX1 and PIN1 polarity defects and AUX1 aggregation.
Immunocytochemistry imaging is shown in **(A)** to **(C)**, **(F)**, and **(G)**, and live-cell imaging of epidermis cells is shown in **(D)** and **(E)**. The localization of AUX1:HA is shown in red, and that of PIN1 is shown in green in **(F)** and **(G)**. Arrowheads indicate ectopic localization. Bars = 10 μm.

agglomeration was observed (Figure 6F). Similarly, genetic interference with sterol composition in the *orc* mutant leads to changes in PIN polarity (Willemsen et al., 2003) and AUX1 localization, also resulting in lateral AUX1 agglomeration (Figure 6G).

Thus, pharmacological and genetic interference with sterols leads to defects in PIN1 and AUX1 polarity and agglomeration of AUX1, suggesting that AUX1 trafficking in protophloem cells strictly requires sterol function. The alternative possibility, that sterol depletion–based mislocalization of PIN proteins leads to alterations in auxin distribution and this, in turn, affects AUX1 trafficking, is unlikely because pharmacological inhibition of auxin transport does not have comparable effects on AUX1 localization (Figure 5E).

DISCUSSION

Mapping the AUX1 Trafficking and Polar Targeting Pathways

The combination of pharmacological, genetic, and live-cell imaging approaches in our studies have revealed a pathway for subcellular trafficking and polar targeting of the AUX1 auxin influx carrier. In protophloem cells, localization of AUX1 is polarized to some extent, with increased amounts of protein found at the upper (apical) PM (Swarup et al., 2001). In addition to this cell surface pool, a smaller proportion of AUX1 is also located intracellularly in the Golgi apparatus and endosomes. The Golgi apparatus–localized AUX1 most likely represents newly synthesized protein, consistent with the finding that the intracellular pool, as well as the recovery of AUX1 at the PM, decreases after the inhibition of protein synthesis. By contrast, the endosomal pool of AUX1 is possibly related to the constitutive recycling of AUX1 to and from the PM. The FRAP analysis of live cells showed an intensive exchange of AUX1 between the PM and the intracellular pool and that this exchange is independent of the synthesis of new protein. It has emerged from multiple studies that in plants, the vesicular motility in interphase cells is based mainly on the actin cytoskeleton (Geldner et al., 2001; Voigt et al., 2005). In accordance, the intracellular AUX1 dynamics strictly require an intact actin cytoskeleton but are only marginally, if at all, dependent on microtubules. Furthermore, the maintenance of the apical PM localization of AUX1 is dependent on the actin cytoskeleton and the sterol composition of the membranes. Unlike PIN1, this constitutive trafficking cannot be visualized using the vesicle trafficking inhibitor BFA, as the delivery of AUX1 to the PM can occur independently of BFA-sensitive ARF GEFs, including ARF GEF GNOM. On the other hand, the subcellular dynamics of the intracellular AUX1 pool requires an as yet unknown BFA-sensitive ARF GEF, as demonstrated by the aggregation of the intracellular endosomal and Golgi apparatus AUX1 pools into BFA compartments. This suggests a complex action of BFA-sensitive and -resistant ARF GEFs at different steps of subcellular AUX1 trafficking and explains the apparent discrepancy that AUX1 trafficking is BFA-sensitive (Grebe et al., 2002) yet auxin influx is BFA-insensitive, in contrast with auxin efflux (Morris and Robinson, 1998).

AUX1 and PIN Trafficking Pathways Are Distinct

Our studies have revealed a constitutive movement of the AUX1 auxin influx component between the PM and the intracellular endosomal pool. Such dynamics share important features with PIN proteins, the constitutive cycling components of auxin efflux. PIN1 trafficking, which, like AUX1 trafficking, can be polar or nonpolar, depending on the cell type (Friml et al., 2002a, 2002b), occurs along the actin cytoskeleton rather than along microtubules (Geldner et al., 2001) and is dependent on the sterol composition of the membranes (Willemsen et al., 2003). The trafficking of the PIN1 vesicles to the PM requires the action of the BFA-sensitive endosomal ARF GEF GNOM (Geldner et al., 2003). Normally, the equilibrium of the cell surface and intracellular pools of PIN1 protein is shifted in favor of the PM pool, so the

intracellular PIN1 is difficult to visualize (Paciorek et al., 2005). By contrast, AUX1 can be more easily detected in intracellular endomembranes consisting partly of Golgi apparatus and endosomes. This population of AUX1 is targeted in a GNOM-independent manner and also in a BFA-resistant manner, at least in trafficking to the PM (Figure 7).

Notably, AUX1 targeting is more sensitive than PIN targeting to interference with the actin cytoskeleton and its dynamics. In addition, a sterol membrane composition is important for AUX1 delivery, as genetic or chemical interference with sterols leads to the retrieval of AUX1 from the PM and its aggregation. Such a sterol–AUX1 relationship is reminiscent of the sterol dependence of apical targeting in epithelial cells. There, selected proteins associate with lipid rafts during apical targeting, with rafts consequently acting as apical sorting platforms (Schuck and Simons, 2004). In animal cells, filipin has been used to specifically interfere with the formation of caveolae and the subsequent inhibition of clathrin-independent endocytosis (Nabi and Le, 2003). Further investigation will clarify whether filipin effects on AUX1 targeting underlie the same caveolae-like endocytosis mechanism in plant cells. In conclusion, the different cell biological requirements of delivery to the PM show that PIN proteins and AUX1 use distinct trafficking pathways. Furthermore, the secretory pathway of the newly synthesized AUX1 from the endoplasmic reticulum is distinct from the PIN1 secretory pathway, as demonstrated by analysis of the role of the endoplasmic reticulum–resident AXR4 protein (Dharmasiri et al., 2006). Thus, AUX1 trafficking represents a novel pathway for the delivery and constitutive trafficking of PM proteins in plants. It seems that AUX1 trafficking also uses similar mechanisms in cell types in which AUX1 is not polarly localized. This suggests that the observed differences in the targeting mechanism reflect specific differences between AUX1 and PIN1 targeting rather than a general difference between apical and basal targeting pathways in plants.

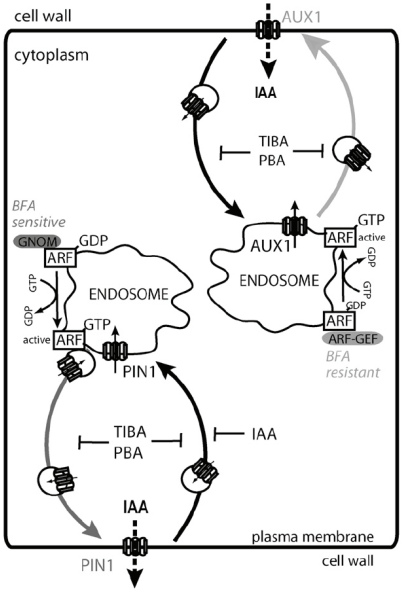

Figure 7. Model of Distinct Pathways for the Constitutive Trafficking of AUX1 and PIN1.

PIN1 and AUX1 are targeted in protophloem cells to distinct polar domains. Both proteins display actin-dependent, TIBA/PBA-sensitive constitutive trafficking between endosomes and the PM, although AUX1 trafficking is more sensitive to TIBA/PBA and actin depolymerization. PIN1 delivery to the PM requires BFA-sensitive, endosomal ARF GEF GNOM, whereas AUX1 trafficking depends on another BFA-resistant ARF GEF. In addition, there is another endosomal, BFA-sensitive ARF GEF (not indicated here) whose inhibition by BFA leads to the aggregation of AUX1-bearing endosomes. The arrows associated with AUX1 and PIN1 proteins show the orientation of these proteins with regard to the direction of transport. It is unclear, however, whether AUX1 and PIN1 also can be active when internalized. IAA, indole-3-acetic acid.

Functional Relevance of AUX1 and PIN Trafficking

We conclude from our studies that AUX1 and PIN1 proteins are trafficked by distinct pathways (Figure 7). Independent targeting of AUX1 and PIN1 provides several functional advantages. First, AUX1 and PIN1 proteins can adopt distinct patterns of localization in the same cell or different cell types to fine-tune polar auxin transport for a particular developmental purpose. For example, AUX1 and PIN1 are targeted to opposite ends of protophloem cells to facilitate the intercellular transport of auxin from the phloem to the root apical meristem. By contrast, colocalization of AUX1 and PIN1 at the same side of L1 cells on the flanks of the shoot apical meristem is hypothesized to create an auxin maximum at this position, demarcating the position of a new leaf primordium (Reinhardt et al., 2003). Second, targeting AUX1 and PIN1 by distinct pathways provides the opportunity to regulate their trafficking independently. For example, PIN recycling is regulated by auxin (Paciorek et al., 2005), whereas AUX1 trafficking seems to be unrelated to auxin. This distinction allows positive feedback by increased intracellular auxin levels on auxin efflux but not influx. This homeostatic mechanism appears entirely logical, as it does not interfere with the AUX1-dependent redistribution of auxin between tissues, which was recently demonstrated to be critical for root gravitropism (Swarup et al., 2005). Hence, the independent regulation of the trafficking of auxin influx and efflux components enables the fine-tuning of polar auxin transport in response to multiple cues and provides an additional level of regulation of this crucial physiological process.

In conclusion, our results provide evidence for the existence of different pathways for the polar targeting of apical and basal cargos in plant cells. Additionally, they also provide AUX1 and PIN1 as model substrates for the further molecular analysis of these distinct trafficking pathways.

METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana plants and seedlings (ecotype Columbia) were grown in growth chambers under long-day conditions at 23°C. Experiments were performed on 5-d-old seedlings grown on vertically oriented plates containing *Arabidopsis* medium (half-strength Murashige and Skoog agar and 1% sucrose, pH 5.8). Incubation of seedlings with various chemicals was performed on 24-well cell culture plates containing the indicated concentrations of chemicals in *Arabidopsis* medium. If not indicated differently, the following conditions were used. Conditional pretreatment for 0.5 h with the first drug was followed by 2 to 3 h of concomitant second drug treatment. Control treatments contained equal amounts of solvent (DMSO or ethanol). The following mutants and transformants have been described previously: *ST:YFP* (Grebe et al., 2003); BRI1:GFP (Russinova et al., 2004); AUX1:HA (Swarup et al., 2001); AUX1:YFP (Swarup et al., 2004); *GNOM*^{M696L}*-myc* (Geldner et al., 2003); and *orc* (Willemsen et al., 2003).

Expression and Localization Analysis

Whole-mount immunofluorescence preparations were assembled as described (Friml et al., 2003). The rabbit anti-PIN1 polyclonal antiserum was raised against amino acids 288 to 452 of PIN1 and used previously for PIN1 localization in tissue sections (Benková et al., 2003; Reinhardt et al., 2003). For whole-mount immunolocalization in roots, immunoglobulins from the crude serum were precipitated by saturated $(NH_4)_2SO_4$ solution (2:1) and dialyzed against PBS. The purified fraction was diluted 1:1000. Other antibodies were diluted as follows: anti-GFP (1:300; Molecular Probes), anti-HA (1:600; SantaCruz), anti-PEP12 (1:200; da Silva Conceicao et al., 1997), anti-ARF1 (1:1000; Pimpl et al., 2000); anti-At γ-COP (1:1000; Movafeghi et al., 1999), and anti-KNOLLE (1:500; Lauber et al., 1997). Fluorescein isothiocyanate– and Cy3-conjugated anti-rabbit secondary antibodies (Dianova) were diluted 1:500, and 1:600, respectively, and YFP was visualized in 5% glycerol without fixation (live-cell imaging). For confocal laser scanning microscopy, a Leica TCS SP2 microscope was used. Images were processed in Adobe Photoshop cs. Profile analyses of the BFA compartment were performed with Leica Confocal Software. For quantification of colocalization, intracellular signals in protophloem cells were marked ($n = 6$), and total pixels of both channels and merged pixels were counted (phyton-based). Subsequently, the percentage of overlaying signal for both proteins was determined, and statistical analysis was performed using Microsoft Office Excel 2003.

Live-Cell Imaging and FRAP Analysis

Live-cell imaging and FRAP analysis were performed using a confocal microscope (model TCS SP2; Leica) equipped with an He-Cd laser and an argon laser (which provides excitation at 514 nm for YFP). For the photobleaching experiment, a region of interest was selected for scans using the Leica FRAP procedure. YFP images before and after scans were collected. All FRAP analyses were performed with Leica Confocal Software.

Accession Numbers

Arabidopsis Genome Initiative locus identifiers for the genes mentioned in this article are as follows: ARF1 (At1g23490), AUX1 (At2g38120), BRI1 (At4g39400), γ-COP/SEC21p (At4g34450), GNOM (At1g13980), KNOLLE (At1g08560), PEP12 (At5g16830), PIN1 (At1g73590), and SMT1 (At5g13710).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Movie 1. Dynamics of AUX1-YFP in *Arabidopsis* Lateral Root Cap Cells.

Supplemental Movie 2. TIBA Inhibits the Dynamics of AUX1-YFP in *Arabidopsis* Lateral Root Cap Cells.

Supplemental Movie 3. Auxin Does Not Visibly Interfere with the Dynamics of AUX1-YFP in *Arabidopsis* Lateral Root Cap Cells.

Supplemental Figure 1. The PVC Marker PEP12 Does Not Colocalize with the Endosomal Marker in *Arabidopsis*.

Supplemental Figure 2. Evaluation of the Colocalization of AUX1:HA and a Subcellular Marker.

Supplemental Figure 3. Intensity Profile Analysis of AUX1 and Subcellular Markers in BFA Compartments.

Supplemental Figure 4. FRAP Charts of AUX1 Dynamics.

Supplemental Figure 5. Oryzalin Does Not Interfere with AUX1/PIN1 Targeting.

Supplemental Figure 6. Effects of Auxin Transport Inhibitors on AUX1 in Epidermis Cells.

Supplemental Figure 7. Filipin Effects on AUX1 in Epidermis and Lateral Root Cap Cells.

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Chapter 3

Result part II:

ARF GEF-Dependent Transcytosis and Polar Delivery of PIN Auxin Carriers in *Arabidopsis*

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Report

ARF GEF-Dependent Transcytosis and Polar Delivery of PIN Auxin Carriers in *Arabidopsis*

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Summary

Cell polarity manifested by the polar cargo delivery to different plasma-membrane domains is a fundamental feature of multicellular organisms. Pathways for polar delivery have been identified in animals; prominent among them is transcytosis, which involves cargo movement between different sides of the cell [1]. PIN transporters are prominent polar cargoes in plants, whose polar subcellular localization determines the directional flow of the signaling molecule auxin [2, 3]. In this study, we address the cellular mechanisms of PIN polar targeting and dynamic polarity changes. We show that apical and basal PIN targeting pathways are interconnected but molecularly distinct by means of ARF GEF vesicle-trafficking regulators. Pharmacological or genetic interference with the *Arabidopsis* ARF GEF GNOM leads specifically to apicalization of basal cargoes such as PIN1. We visualize the translocation of PIN proteins between the opposite sides of polarized cells in vivo and show that this PIN transcytosis occurs by endocytic recycling and alternative recruitment of the same cargo molecules by apical and basal targeting machineries. Our data suggest that an ARF GEF-dependent transcytosis-like mechanism is operational in plants and provides a plausible mechanism to trigger changes in PIN polarity and hence auxin fluxes during embryogenesis and organogenesis.

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Results and Discussion

PIN Targeting Utilizes Distinct ARF GEF-Dependent Apical and Basal Pathways

Previous studies have suggested that the polar delivery of auxin efflux and influx components occurs via different pathways [4, 5]. The fungal toxin brefeldin A (BFA) has been shown to specifically inhibit a subclass of ARF GEFs. The recycling of PIN auxin efflux carriers from endosomes to the plasma membrane is BFA sensitive, as reflected by the accumulation of PINs in aggregated endosomal BFA compartments in response to BFA [6, 7].

We observed that the localization of PIN proteins at the apical (shoot-apex-facing) and basal (root-apex-facing) sides of *Arabidopsis* root cells differed dramatically in their sensitivity to the BFA treatment. Within 60 min, basal cargoes, such as PIN1 in the stele or PIN2 in the young cortex cells, were almost completely internalized from the plasma membrane into BFA compartments, whereas most of the apically localized PIN2 in the epidermis remained at the plasma membrane in addition to its intracellular accumulation (Figures 1A–1D). To address whether apical and basal targeting display differential sensitivities to BFA in the same cell type, we used transgenic lines to target two different, but both functional, versions of PIN1 to opposite sides of epidermal cells [2]. After short-term BFA treatments, the basal PIN1-HA (Figure 1E) was almost completely internalized within 60 min of BFA treatment (Figure 1F), whereas the apical PIN1-GFP-3 remained to a large degree at the apical plasma membrane (Figures 1G and 1H). Preferential BFA-induced internalization of the basal cargoes was particularly apparent when PIN1-HA (basal) and endogenous PIN2 (apical) were simultaneously visualized in the same epidermal cells (Figures 1I and 1J).

These experiments show that the apical and basal targeting in plants involves different sets of ARF GEF proteins. Basal targeting strictly requires BFA-sensitive ARF GEFs, whereas apical delivery is largely insensitive to BFA. Consequently, apical and basal pathways in plants are distinct and operate in parallel in the same cell.

Inhibition of BFA-Sensitive ARF GEFs Leads to Recruitment of Basal PIN Cargoes into the Apical Pathway

Next, we addressed whether the apical and basal targeting pathways are interconnected and whether they can alternatively be used by the same cargoes. The inhibitory effect of BFA was exploited to interfere preferentially with basal, but not apical, PIN targeting.

After prolonged BFA treatments, the internalized PIN1 in BFA compartments decreased and PIN1 gradually appeared at the apical plasma membrane (Figures 2A–2C). For PIN2 in the cortex cells, BFA incubations as short as 2 hr were sufficient to induce a pronounced basal-to-apical polarity shift (Figure 2D). Longer treatments or the use of higher BFA concentrations confined the localization of both PIN1 and PIN2 to a restricted region in the middle of the apical membrane, designated "superapical domain" (Figures 2C and 2E). In contrast to basal cargoes, apical cargoes, such as PIN2 or PIN1-GFP-3, in the epidermis stayed at the apical plasma membrane

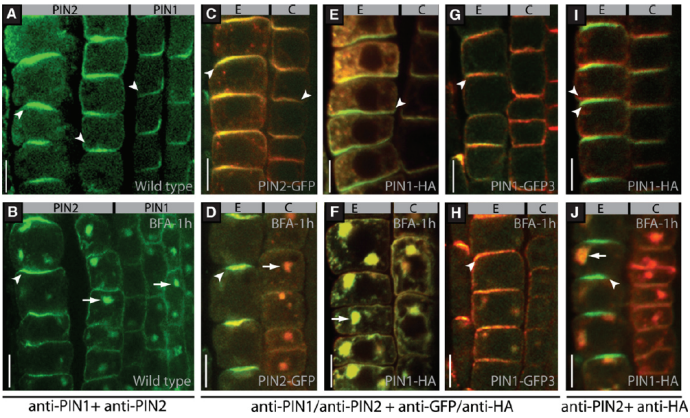

Figure 1. Distinct Apical and Basal ARF GEF-Dependent Targeting of PIN Proteins

(A and B) Apical localization of PIN2 in the epidermis; basal localization of PIN2 in the cortex and PIN1 in the stele and endodermis cells (A). BFA treatment (50 μM) leads to a strong internalization of the basal PIN1 and PIN2 but not apically localized PIN2 (B).

(C and D) Apical localization in the epidermis, but basal in the cortex cells of PIN2-GFP (C). In cortex cells, basal PIN2-GFP rapidly internalizes after BFA incubation, whereas in epidermal cells it displays BFA-resistant plasma-membrane localization (D).

(E and F) Basal localization in epidermal and cortex cells of PIN1-HA (E). Basally localized PIN1-HA shows a rapid BFA-dependent internalization in both cell types (F).

(G and H) PIN2-like distribution in epidermal (apical) and cortex (basal) cells of PIN1-GFP-3 (G). Apical PIN1-GFP-3 localization in the epidermis is resistant to BFA treatment (H).

(I and J) Simultaneous visualization of apically localized PIN2 (green) and basal PIN1-HA (red) in epidermal cells (I); PIN1-HA internalizes completely after BFA treatment, whereas PIN2 largely remains at the apical plasma membrane (J).

Arrows depict PIN proteins in the BFA compartments and arrowheads PIN polarity. E denotes epidermis; C denotes cortex. Scale bars represent 10 μm.

even after prolonged BFA treatment (Figures 1H and 1J; Figure S1E available online). Notably, these effects were specific for polar cargoes (Figures S1A and S1B) and were not based on a mere disappearance of the BFA compartments (Figures S1C–S1E).

Recently, it has been shown that *Arabidopsis* Sorting Nexin1 (SNX1)-dependent and Vacuolar Protein Sorting 29 (VPS29)-dependent pathways are involved in PIN targeting [8, 9]. However, BFA-induced PIN apicalization was still observed in corresponding *snx1* and *vps29* mutants (Figures S2A–S2C); this result does not support a role of these regulators at the intersection of ARF GEF-dependent apical and basal pathways.

Taken together, these data provide the mechanistic insight that inhibition of BFA-sensitive ARF GEFs leads to a recruitment of basal cargoes into the apical targeting pathway. This suggests that the apical and basal targeting pathways are interconnected and can be used alternatively by the same polar cargoes.

Inhibition of ARF GEF GNOM Is Sufficient to Recruit Basal PIN Cargoes into the Apical Pathway

Next we addressed which BFA-sensitive ARF GEFs are required for the BFA-induced basal-to-apical shift in PIN polarity. The endosomal ARF GEF GNOM was an obvious candidate because GNOM is sensitive to BFA and has been shown to be involved in the endosome-to-plasma membrane targeting of different cargoes, including PIN1 [7].

We used transgenic lines in which the wild-type GNOM had been replaced by an engineered BFA-resistant version (*GNOM*^{M696L}) [7]. In these lines, BFA-dependent apicalization of PIN1 or PIN2 in cortex cells did not occur (Figures 2F and 2G), showing that it is the specific inhibition of GNOM by BFA that leads to the BFA-induced PIN apicalization. Next, we examined partial loss-of-function alleles of *GNOM* (*gnom*^{R5} and *van7*) that initially form correctly patterned roots after germination [10, 11]. Basal cargoes, such as PIN1 in the stele and PIN2 in the cortex cells, showed apicalization reminiscent of long-term BFA treatment (Figures 2H and 2I). On the other hand, the localization of apical cargoes, such as PIN2 in epidermal cells, and nonpolar cargoes, such as plasma membrane H^+-ATPase, were unaffected in either of these *gnom* alleles (Figure 2I; data not shown). In analogy to BFA treatments, we observed cells with "superapical" PIN localization in untreated *gnom* alleles (Figure 2I). Notably, BFA treatments led to a reduced PIN accumulation in BFA compartments and to a faster and more pronounced PIN apicalization in *gnom* mutant roots compared to wild-type roots (Figures S3A and S3B).

These results show that pharmacological or genetic inhibition of the GNOM ARF GEF is sufficient to recruit basal cargoes into the apical pathway. In addition to its general function in endosome-to-plasma membrane trafficking for polar and nonpolar cargoes [7, 12], GNOM obviously plays a role specifically in basal targeting, whereas apical targeting is independent of GNOM and possibly requires BFA-resistant ARF GEFs.

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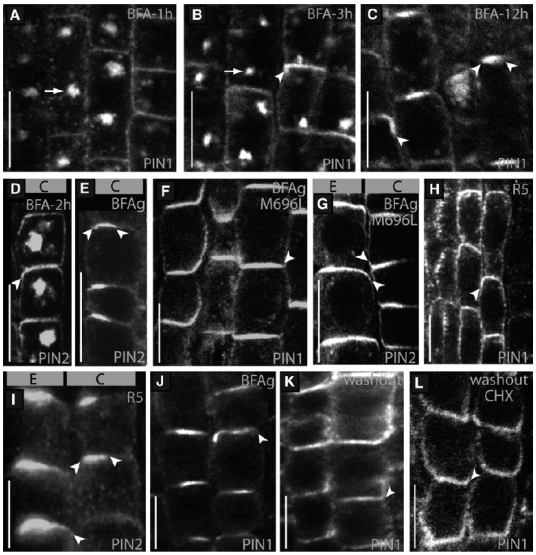

Figure 2. Apicalization of Basal Cargoes after Inhibition of BFA-Sensitive ARF GEF GNOM

(A–C) PIN1 targeting after BFA (50 μM) treatment. In the stele, PIN1 almost completely internalizes after 1 hr (A). Prolonged BFA incubation (2–3 hr) leads to a decrease of PIN1 in BFA compartments and its gradual occurrence at the apical plasma membrane (B). After 12 hr of BFA treatment, PIN1 is absent in the BFA compartment and completely confined to the apical plasma membrane (C).

(D and E) Short-term BFA treatment (50 μM; 2 hr) results in a basal-to-apical shift of PIN2 in cortex cells (D), whereas BFA (25 μM)-germinated seedlings display super apical PIN2 localization in cortex cells (E).

(F and G) The engineered BFA-resistant *GNOM*^{M696L} seedlings do not show the BFA-induced basal-to-apical shift of PIN1 in the stele (F) and of PIN2 in the cortex cells (G) even after several days of BFA exposure.

(H and I) Basal-to-apical shift of PIN1 (H) and PIN2 (I) in untreated root cells of partial loss-of-function *gnom*^{R5} mutant.

(J–L) Apical localization of PIN1 in wild-type seedlings germinated on BFA (J). Translocation of PIN1 to the basal plasma membrane after BFA removal (2 hr in liquid MS) (K) and in the presence of the protein-biosynthesis inhibitor cycloheximide (50 μM; 2 hr) (L).

Immunocytochemistry of anti-PIN1 (A–C, F, H, J–L) and anti-PIN2 (D, E, G, I); arrowheads depict polar localization of PIN proteins. E denotes epidermis; C denotes cortex. Scale bars represent 10 μm.

Inhibition of ARF GEF GNOM Reveals Directional Transcytosis of PIN Proteins between Apical and Basal Polar Domains

The BFA-induced basal-to-apical shift of PIN proteins was fully reversible, and the re-establishment of basal PIN localization after BFA removal was independent of protein biosynthesis (Figures 2J–2L). Moreover, fluorescent recovery after photobleaching (FRAP) of PIN2-GFP at the basal cell side after BFA removal depended on the apical pool of PIN2-GFP (Figure 3A; Figure S4A). These results suggest that the same cargo molecules were translocating between apical and basal cell sides.

To test this scenario directly, we used the green-to-red photoconversion capability of the fluorescent tag EosFP [13], enabling us to trace the dynamics of PIN2-EosFP in vivo. In *35S:PIN2-EosFP*-overexpressing lines, PIN2-EosFP localized strongly to the apical as well as, to some extent, to the basal side of epidermal cells [13]. We observed internalization of formerly basally localized PIN2-EosFP after BFA treatment and its subsequent appearance at the upper cell periphery, demonstrating PIN2 translocation from the basal to the apical cell side (Figure 3B). In polarized cells, BFA treatment typically leads to formation of two BFA compartments per cell positioned closer to either the apical or basal side of the cell [6]. We observed a rapid translocation of PIN2-EosFP from the basal to the apical compartment (Figures 3C and 3D). In contrast, PIN2-EosFP from the apical compartment translocated predominantly to the apical plasma membrane and never to the basal BFA compartment (Figures 3C and 3D). As expected, this basal-to-apical shift of PIN2-EosFP was compromised in the *GNOM*^{M696L} line expressing the BFA-resistant GNOM version (Figures S4B and S4C). Thus, both the PIN2-GFP FRAP and PIN2-EosFP photoconversion in the presence of BFA or after its removal reveal unidirectional translocation of PIN2 molecules between apical and basal cell sides.

Next we investigated whether dynamic translocation of PIN protein in root epidermal cells also occurs independently of BFA treatments. In our experimental conditions, it was difficult to visualize translocation of activated PIN2-EosFP in interphase cells (Figures S4D and S4E). However, we detected substantial retargeting of PIN2-EosFP from the apical and basal cell sides to the newly completed plasma membrane after cell division (Figures 3E and 3F, and Figures S5A and S5B). This observation extends on the recent finding on the importance of sterol-dependent endocytosis for PIN2 polarity re-establishment after cytokinesis [14] and confirms our hypothesis that dynamic translocation of PIN proteins between apical and basal cell sides occurs in vivo.

In animal cells, movement of cargoes, such as the transferrin receptor, between separated plasma-membrane domains is termed transcytosis [15]. Because our experiments provide an in vivo demonstration of a comparable event in plant cells, we propose the use of the same term. In further analogy to animal cells [16, 17], our findings reveal that the apical-to-basal transcytosis of PIN proteins involve BFA-sensitive ARF GEFs. Thus, basal targeting of PIN proteins in polarized plant cells and the basolateral localization of transferrin receptors in animal cells are remarkably analogous and might possibly follow an evolutionarily conserved principle.

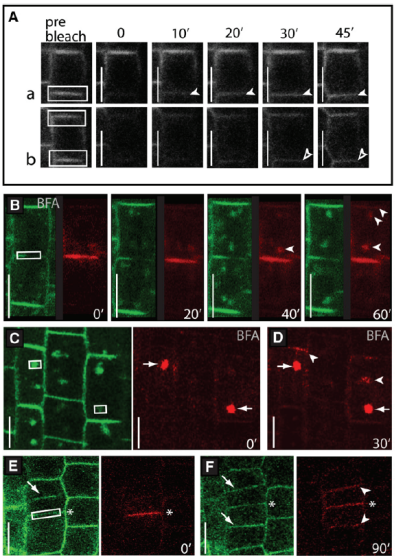

Figure 3. BFA-Dependent Apical-Basal Transcytosis of PIN2

(A) FRAP experiments in the presence of cycloheximide (50 μM; 45 min pretreatment) illustrating the apical-to-basal transcytosis of PIN2 in cortex cells. After long-term BFA treatment (50 μM; 12 hr), apicalized PIN2-GFP in the cortex cells rapidly recovers at the basal plasma membrane following BFA removal (A_R). This recovery is compromised after additional photobleaching of the apical pool of PIN2 GFP (A_b).

(B) Representative pictures illustrating time lapse of PIN2-EosFP (green) and photoconverted (red) in the presence of BFA (50 μM) 0, 20, 40, and 60 min after photoconversion. Gradual translocation of photoconverted PIN2-EosFP from the basal to the apical cell sides through the BFA compartments.

(C and D) BFA (50 μM; 1 hr)-treated PIN2-EosFP-expressing cells (green) 0 (C) and 30 min (D) after photoconversion (red). Photoconverted PIN2-EosFP translocated from the BFA compartment to the apical cell side or from the basally localized BFA compartment to the apically localized compartment.

(E and F) PIN2-EosFP (green) and activated PIN2-EosFP (red) shortly (E) and 90 min (F) after activation in untreated root epidermal cells. Freshly divided cells show substantial PIN2 translocation to the newly built cell sides (F).

Live-cell imaging of PIN2-FP (A–F); arrows indicate activated BFA compartments (C and D) or freshly divided cells (E and F), asterisks depict activated cell side, and arrowheads the translocated PIN2-FP. Scale bars represent 10 μm.

GNOM Mediates the Dynamic Establishment of Basal PIN Polarity during Embryogenesis and Postembryonic Organogenesis

Our data suggest that a GNOM-dependent transcytosis mechanism can be utilized to re-establish basal PIN localization in polarized cells. In addition to a set PIN polar localization in different cell types [18–20], basal PIN1 localization is established dynamically during various developmental processes including formation of embryonic axes [21], organogenesis [11, 22], and formation of vasculature [23, 24]. All these events also require GNOM, which prompted us to re-examine selected developmental events from a transcytosis point of view.

The earliest known rearrangement of PIN polarity is the switch from apolar to basal localization of PIN1 in the inner cells of the *Arabidopsis* 32-cell-stage proembryo; this switch establishes directional auxin flow to the region of the future root meristem [21]. We inhibited ARF GEF function at relevant stages by using in vitro cultures of *Arabidopsis* embryos within their ovules [25]. Embryos that were cultivated in the presence of BFA failed to polarize PIN1 to the basal side of provascular cells, but preferentially showed PIN1 at the apical cell side (Figure 4B). Analogous to roots, apical or nonpolar cargoes remained unaffected (Figure 4B; data not shown). In contrast, in BFA-resistant *GNOM*^{M696L} embryos, PIN1 polarized normally before the 64-cell stage in the presence of BFA (Figure 4C). Consistently with these observations, the earliest PIN1 polarity defect in *gnom* mutant embryos is the failure of early PIN1 polarization to the basal sides of provasculature cells, whereas the apical PIN1 localization in outer layers remained largely unaffected (Figures 4D and 4E). In line with the role of PIN proteins in directional auxin distribution [2], apicalization of PIN1 during embryogenesis affected the auxin distribution and apical-basal embryo patterning (Figures S6A–S6D). Our data substantiate previous findings [21, 26] on the role of GNOM ARF GEF in embryonic patterning and suggest that the initial function of GNOM in embryogenesis is the early establishment of basal polarization of PIN1 in provascular cells.

Similar to the situation in embryogenesis, the development of various organ primordia is accompanied by dynamic rearrangements of PIN polarity [22, 27, 28]. In lateral root primordia, PIN1 localization undergoes a polarity switch from the anticlinal to the outer periclinal cell side that represents the new basal (lateral-root-apex-facing) side [11, 22]. This polarity switch of PIN1 occurs early between stages I and III of primordium development (Figure S6E). Pharmacological [22] or genetic [11] inhibition of the ARF GEF function interferes with PIN1 polar localization during primordia formation and, consequently, with their development. We observed that inhibition of the ARF GEF function by BFA did not primarily alter the advent of PIN1 polarity shift to the periclinal side, but that PIN1 localized predominantly to the opposite, inner periclinal cell side (Figures 4F and 4G). In contrast, no defects in PIN1 relocation or auxin distribution were observed in the BFA-resistant *GNOM*^{M696L} line after BFA incubation (Figures 4J and 4K). These results support the assumption that GNOM mediates dynamic PIN polarity changes to the outer periclinal cell side, which are required for the establishment of new growth axes during de novo organ formation. Notably, this polarization event defines the new basal cell side of the future organ.

Collectively, our findings show that GNOM is required for switches of PIN polarity to the basal cell side that are occurring during embryonic and in postembryonic organ formation. Evident analogies between these events and the transcytosis-like mechanism detected in polarized root cells suggest that these developmental events are mediated by a GNOM-dependent transcytosis mechanism.

Conclusions

Our data provide novel insights into the mechanism of dynamic subcellular polar delivery of PIN auxin efflux carriers and thus into the control of intercellular auxin flow. We show for the first time that cargoes exemplified by PIN proteins

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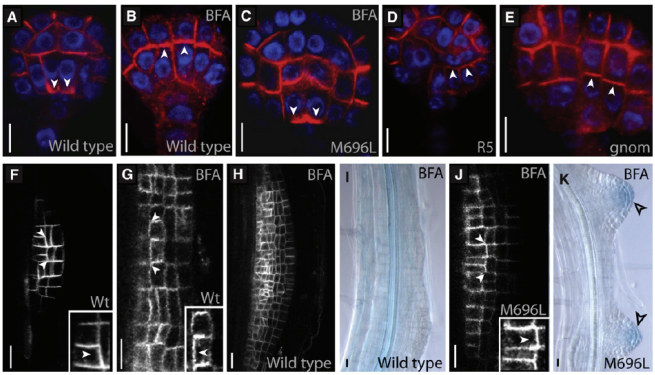

Figure 4. ARF GEF GNOM-Dependent Basal Retargeting of PIN1 in Embryogenesis and Lateral Root Organogenesis

(A–E) PIN1 polarization during apical-basal axis formation. Basal polarization of PIN1 in provascular cells at the 32-cell stage of the *Arabidopsis* embryos (A). BFA-treated (10 μM; 7 days) embryos fail to correctly polarize PIN1 (B). PIN1 translocation to the basal plasma membrane in BFA-resistant $GNOM^{M696L}$ mutants after BFA treatments is shown (C). Partial loss-of-function $gnom^{R5}$ mutant embryos display defects in the basal targeting of PIN1 (D). Full-knockout *gnom* embryos show apical PIN1 localization in all cell types (E).

(F–K) Establishment of an anticlinal-to-periclinal shift in PIN1-GFP localization, pointing toward the presumptive primordium tip during lateral root development (F). BFA-treated (10 μM; 48 hr) roots display predominant inward localization (away from the primordium tip) (G). Unorganized cell division and loss of cellular polarity during progression of lateral root primordia (H) as well as altered auxin response (*DR5:GUS*) distribution (I) after BFA treatments are shown. PIN1-GFP polarized toward the primordia tip after BFA treatment in $GNOM^{M696L}$ (J), resulting in rescued *DR5:GUS* pattern (K).

Immunocytochemical images (A–E) and live-cell imaging of lateral root primordia (F–H and J). Open and filled arrowheads indicate DR5 activity and polar localization of PIN1, respectively. Scale bars represent 10 μm.

can move between different sides of plant cells and by this means change their polar localization. This transcytosis mechanism is realized by a combination of constitutive endocytic recycling [6, 13, 29] and alternative recruitment of cargoes by distinct ARF GEF-dependent apical and basal targeting machineries (Figure S7). The endosomal ARF GEF GNOM plays in this context a specific role in basal recycling. It seems that maintenance of the basal PIN localization in polarized root cells and its dynamic establishment in young embryos or lateral root primordia share a common GNOM-dependent polar-targeting mechanism. In this scenario, the evolutionarily conserved mechanism of GNOM-dependent PIN transcytosis to the basal cell side (Figure S7) would be utilized in plants for a wide range of developmental processes involving rapid changes in PIN polarity. Diverse developmental or environmental [30] cues could thus be integrated to redirect the auxin flow between cells, altogether modulating patterns of auxin-dependent development.

Supplemental Data

Experimental Procedures and seven figures are available at http://www.current-biology.com/cgi/content/full/18/7/526/DC1/.

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Supplemental Data

ARF GEF-Dependent Transcytosis and Polar Delivery of PIN Auxin Carriers in *Arabidopsis*

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Supplemental Experimental Procedures

Used Materials

DR5rev:GFP [S1] and *DR5:GUS* [S2]; *gnom* and *gnom*^{R5} [S3]; *van7* [S4]; *GNOM*^{M696L} [S5]; *PIN2:PIN2-GFP*, *PIN2:PIN1-HA*, and *PIN2:PIN1-GFP-3* [S6]; *BRI1:BRI1-GFP* [S7]; *35S:PIP2-GFP* [S8]; *snx1-1* [S9]; *vps29-3* obtained from NASC [S10]; and *35S:PIN2-EosFP* [S11] have been described previously.

Growth Conditions

Plants were grown on soil or MS plates as described [S12] under a 16-hr-light/8-hr-dark photoperiod at 21°C or 18°C.

Drug Applications and Experimental Conditions

Exogenous drugs were applied by incubation of 5-day-old seedlings in liquid or solid half-strength Murashige and Skoog (MS) medium supplemented with BFA (50 mM stock in dimethylsulfoxide [DMSO]) (10/25/50 μM), cycloheximide (50 mM stock in DMSO) (50 μM), or indole-3-acetic acid (IAA) (10 mM stock in ethanol) (10 μM). Control treatments contained an equivalent amount of solvent (DMSO or ethanol).

For BFA washout experiments (concomitant cycloheximide and BFA pretreatment for 0.5 hr), seedlings were rinsed three times in liquid MS medium (conditionally supplemented with cycloheximide) and subsequently washed in MS medium (conditionally supplemented with cycloheximide) for the indicated time periods. Three independent washout experiments were performed with n > 28 roots, and representative pictures are presented. For in vitro embryo culture [S13], excised ovules were placed on half-strength MS medium containing 2% sucrose, 400 mg/liter glutamine, and 0.3% Phytagel. For treatments, this medium was supplemented with 10 μM of BFA. Plates were kept in the dark at 22°C for up to 7 days. At different time points, embryos were excised from the ovules for microscopic analysis. For all treatments, markers, and mutant-phenotype analyses, control experiments were done in the sister lines, a total number of at least 28 embryos for each treatment were analyzed, and representative images are presented. For in vivo analysis of the development of individual lateral root primordia, 5-day-old seedlings were transferred on slides with a thin layer of half-strength MS medium with 0.5% agarose, supplemented with IAA (5 μM; 48 hr) or IAA/BFA (10 μM/10 μM), and incubated 24–48 hr in a humid chamber. For each treatment, at least 30 lateral primordia (roots n = 10) were analyzed, and representative images are presented. *DR5:GUS* was analyzed by staining with α-glucuronidase (GUS) as described [S14]. For all comparisons, independent experiments were done at least in triplicate with the same significant results. Data were statistically evaluated with Excel, 2003 (Microsoft).

Expression and Immunolocalization Analyses

Whole-mount immunofluorescence was performed as described [S1]. Antibodies were diluted as follows: 1:1000 for rabbit anti-ARF1 (generously provided by D.G. Robinson); 1:500 for mouse anti-PM-ATPase (generously provided by W. Michalke); 1:2000 for rabbit anti-PIN1 [S15]; 1:2000 for rabbit anti-PIN2 (generously provided by C. Luschnig); 1:500 for rabbit anti-GFP (Molecular probes); 1:800 for mouse anti-GFP (Roche); and 1:500 and 1:600 for FITC- and CY3-conjugated anti-mouse and anti-rabbit secondary antibodies (Dianova), respectively. For each genotype or treatment, at least three to five independent experiments were performed, 36 to 60 roots in total were analyzed, and representative images are presented. GFP or EosFP was visualized in 5% glycerol or in chambered cover glass (Nunc) submerged with solid MS media without fixation for live-cell imaging. For statistical evaluation of ARF GEF GNOM-dependent apicalization, only median scans were analyzed, and for each treatment at least 1000 stele and 400 cortex cells (roots n > 12) were counted. For the photobleaching experiments, 5- to 6-day-old seedlings were mounted in liquid media on slides or in chambered cover glass (Nunc) submerged with solid MS media. A region of interest was selected for scans with the Leica LCS confocal software 2004 (Leica) FRAP procedure as previously described [S16]. GFP images before and after scans were collected. Z stack imaging was used to confirm bleached region and fluorescent recovery after 1 hr. A total number of four cells were analyzed (n = 4), and representative images and unpaired Student's t test evaluation are presented. 35S:PIN2-EosFP analysis was performed in Col-0 background that, similar to the overexpression of 35S:PIN2-GFP [S17], displayed wavy-root phenotype. Moreover, 35S:PIN2-EosFP reduced agravitropic root phenotype of *pin2* mutants. EosFP expressing 5- to 6-day-old seedlings were mounted in liquid media on slides or in chambered cover glass (Nunc) submerged with solid MS media and were analyzed as described [S11]. For BFA-treatment experiments, a total number of 32 cells were analyzed (roots n = 8) and followed over time, and representative images are presented in the figures. For analysis of untreated cells, 33 interphase and nine cytokinetic cells (roots n = 14 and n = 8, respectively) were imaged before, shortly after, and 90 min after activation. Representative pictures are presented.

Fluorescent intensity was measured by ImageJ or Leica confocal software. For confocal laser-scanning microscopy, single scans were obtained with Leica TCS SP2 AOBS or Olympus FV10 ASW. Images were processed in Adobe Photoshop and Illustrator CS2 (Adobe).

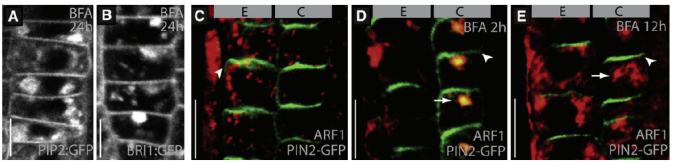

Figure S1. BFA-Dependent Apicalization Is Specific to Polar Cargoes

(A and B) No apicalization but continuous localization in BFA compartments of aquaporin PIP2-GFP (A) or brassinosteroid receptor BRI1-GFP (B) even after prolonged BFA incubations (50 μM; 24 hr) suggests that only polar cargoes can be recruited into the BFA-resistant apical targeting pathway.

(C–E) PIN2-GFP (green) and endosomal ARF1 (red) localization in untreated root cells (C). PIN2-GFP is apically and basally targeted in epidermal and cortex cells, respectively. PIN2-GFP shows a BFA-dependent (50 μM; 2 hr) apicalization in cortex cells and partial colocalization with endosomal ARF1 in BFA compartments (D). Decrease of PIN2-GFP in BFA compartments but continuous accumulation of ARF1 after prolonged BFA incubations (12 hr) (E) illustrates that the gradual disappearance of PIN proteins in BFA compartments is not due to a disappearance of the BFA compartments themselves.

Live-cell imaging (A and B) and immunolocalization (C–E); arrows mark BFA compartments, and arrowheads mark polar localization of PIN proteins. E denotes epidermis; C denotes cortex. Scale bars represent 10 μm.

S2

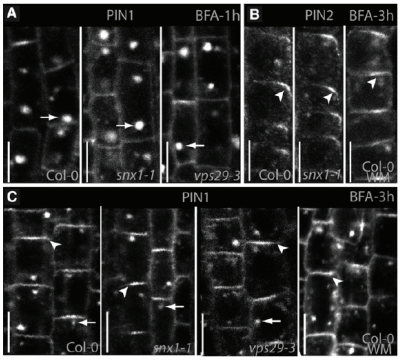

Figure S2. SNX1- and VPS29-Independent Basal-to-Apical Shift of PIN Proteins

(A–C) Representative images of anti-PIN1 (A and C) and anti-PIN2 (B) immunolocalization after short-term BFA (50 μM) treatments in wild-type and putative retromer-complex mutants. *snx1* and *vps29* do not show changed internalization after 1 hr treatment by BFA (A), whereas 2–3 hr treatments lead to wild-type-like apical PIN2 localization in young cortex cells in *snx1* mutants or after wortmannin-dependent inhibition of SNX1-labeled prevacuolar compartments (B). BFA treatments lasting 3–4 hr lead to a similar decrease of PIN1 in BFA compartments and gradual appearance at the apical cell side in wild-type, *snx1-1*, *vps29-3*, and wortmannin-treated seedlings (C), suggesting that genetic or pharmacological interference with SNX1 and VPS29 functions at the prevacuolar compartment does not affect BFA-dependent basal-to-apical shift of PINs.
Arrows mark BFA compartments, and arrowheads mark polar localization of PIN proteins. Scale bars represent 10 μm.

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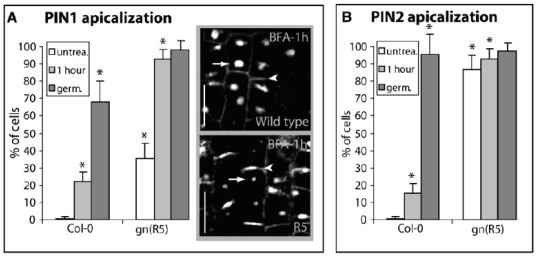

Figure S3. ARF GEF-Dependent Apicalization of Basal PIN Cargoes

(A and B) Apical localization of PIN1 in the stele (A) and of PIN2 in the cortex cells (B) in the presence and absence of BFA in different genetic backgrounds. Inset shows localization of PIN1 after BFA treatment (50 μM; 1 hr) in wild-type and *gnom*^{R5} mutant seedlings (A). Note the higher frequency of apical PIN2 versus PIN1 in *gnom*^{R5}, which may illustrate differential affinity of PIN2 and PIN1 for the apical targeting pathway. Error bars represent average values (± standard deviation [SD]); at least 1000 stele and 400 cortex cells for each treatment or genotype (roots n > 12) were counted; differences between treated and untreated wild-type seedlings and differences among genotypes were highly significant (asterisks) on the basis of unpaired Student's t test evaluation ($p < 0.008$). Arrows mark BFA compartments, and arrowheads mark polar localization of PIN proteins. E denotes epidermis; C denotes cortex. Scale bars represent 10 μm.

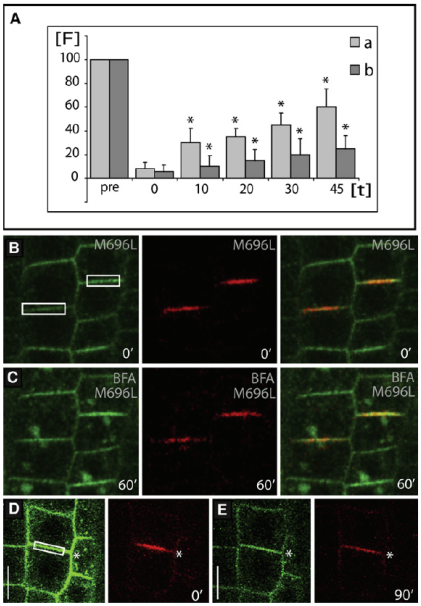

Figure S4. ARF GEF-Dependent Translocation of PIN2

(A) Diagram quantifies the FRAP experiment of four independent cells (n = 4). After long-term BFA treatment (50 μM; 12 hr), apicalized PIN2-GFP in the cortex cells rapidly recovered at the basal plasma membrane following BFA removal in the presence of cycloheximide (A_a). This recovery was significantly compromised after additional photobleaching of the apical pool of PIN2-GFP (A_b). Error bars represent average values (± SD; asterisks indicate $p < 0.05$).

(B and C) PIN2-EosFP (green) and activated PIN2-EosFP (red) in the engineered BFA-resistant *GNOM*^{M696L} seedlings shortly after (B) and 60 min after (C) photoconversion reveals weak PIN2 accumulation following BFA incubation. However, wild-type-like translocation of PIN2-EosFP to the apical cell side was not visible in *GNOM*^{M696L}.

(D and E) PIN2-EosFP (green) and activated PIN2-EosFP (red) in untreated wild-type epidermal interphase cells shortly after (D) and 90 min after (E) photoconversion.

F indicates relative fluorescence; t indicates time in minutes. Live-cell imaging of root cells (B–E). Region of photobleaching or activation is indicated by white boxes; arrowheads depict FRAP; asterisks in (D and E) indicate the activated cell side. Scale bars represent 10 μm.

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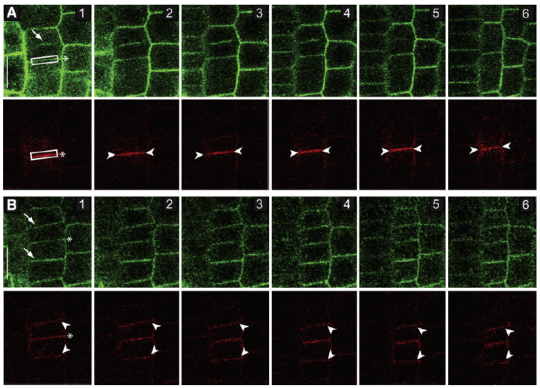

Figure S5. PIN2-EosFP Translocation in Cytokinetic Root Epidermal Cells

(A and B) Representative Z stack series (1 μm steps) of PIN2-EosFP (green) and activated PIN2-EosFP (red) in cytokinetic cells shortly after (A) and 90 min after (B) activation.

Live-cell imaging of epidermal root cells (A and B); arrows indicate newly formed plasma membrane, and arrowheads indicate the translocated PIN2-EosFP; region of activation is indicated by boxes, and asterisks mark the activated cell wall. Scale bars represent 10 μm.

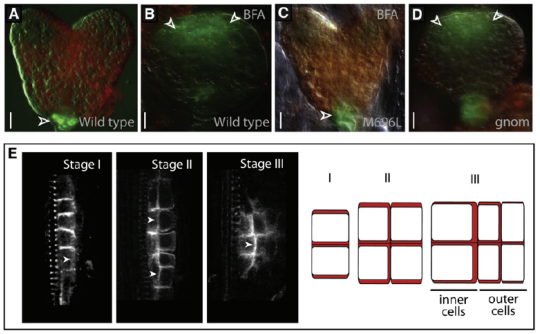

Figure S6. ARF GEF GNOM-Induced Retargeting of PIN1 during Plant Development

(A–D) Auxin-responsive *DR5rev:GFP* expression pattern in wild-type embryos (A). Ectopic apical *DR5* activity in wild-type embryos caused by BFA (10 μM; 7 days) treatment (B) is shown. Rescue of the *DR5* pattern and development in BFA-treated embryos (10 μM; 7 days) of BFA-resistant *GNOM*^{M696L} line (C) is shown. Ectopic apical *DR5* activity in *gnom* mutant embryos (D) is shown.

(E) PIN1 polarity shift from anticlinal-to-outer periclinal cell side early during development of lateral root primordia, suggesting dynamic translocation of PIN1.

Live-cell imaging (A–D); immunolocalization of PIN1 (E); open and filled arrowheads indicate DR5 activity and polar localization of PIN1, respectively. Scale bars represent 10 μm.

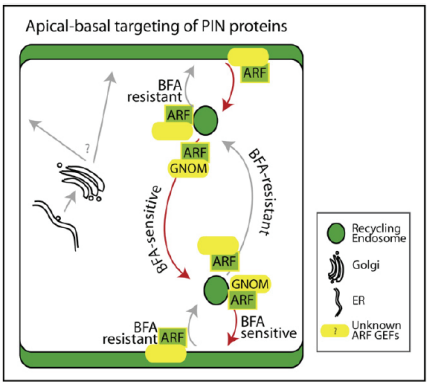

Figure S7. Model for Transcytosis of PIN Proteins

Hypothetical representation of the distinct ARF GEF-dependent apical and basal targeting machineries in plant cells. GNOM activity determines apical-basal targeting of PIN proteins. The depletion of GNOM activity leads to basal-to-apical translocation of PIN molecules. The same polar competent cargo can be recruited by both distinct polar targeting pathways, hence enabling flexible deposition of the polar cargo. Alternative PIN recruitment to distinct ARF GEF-reliant targeting pathways may enable synchronous apical-basal targeting of PIN proteins, redefining of PIN polarity (e.g., counteracting lateral diffusion) or rapid PIN polarity alterations. It is tempting to hypothesize that developmental processes that involve asymmetric auxin distribution could utilize the transcytosis mechanism for triggering changes or maintenance in the PIN polarity and for directing the intercellular auxin flow.

Chapter 4

Result part III:

Cellular and molecular requirements for polar PIN targeting and transcytosis in plants

Cellular and molecular requirements for polar PIN targeting and transcytosis in plants

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Running title: polar auxin carrier trafficking

Summary

The polar, subcellular localization of PIN efflux carriers determines the direction of intercellular auxin flow, thus defining the spatial aspect of auxin signalling. Dramatic, transcytosis-like remobilization of PIN proteins can occur in response to external and internal signals, integrating them into changes in auxin distribution. Here, we examine the cellular and molecular mechanism of polar PIN delivery and transcytosis. The mechanisms of the ARF-GEF dependent polar-targeting and transcytosis are very well conserved among diverse *Arabidopsis* ecotypes, consistent with their fundamental importance in regulating plant development. At the cellular level, we refine previous findings on the role of the actin cytoskeleton in apical and basal PIN targeting, and identify a previously unknown role for microtubules, specifically in basal targeting. PIN protein delivery to different sides of the cell is mediated by ARF-dependent trafficking with a previously unknown complex level of distinct ARF-GEF vesicle trafficking regulators. Our data suggest that alternative recruitment of PIN proteins by these distinct pathways can account for cell type- and cargo-specific aspects of polar targeting, as well as for polarity changes in response to different signals. The resulting dynamic PIN positioning to different sides of cells defines a three-dimensional pattern of auxin fluxes within plant tissues.

Introduction

Plant development is characterized by an exceptional ability to redefine the polarity of whole tissues, in addition to individual cells. Tissue polarization is a necessary component of developmental processes such as *de novo* organogenesis, vascular tissue formation and regeneration. Nonetheless, knowledge on cell polarity and mechanisms of targeted cargo delivery in plants is still limited. Classical physiological and current molecular studies have identified the plant hormone auxin as a key signal whose asymmetric distribution provides positional and directional information for various developmental events including tissue polarization (reviewed in Friml 2003; Tanaka et al., 2006; Willemsen and Scheres, 2004; Leyser et al., 2005). Asymmetric auxin distribution depends on directional (polar) transport between cells by auxin transport proteins, including PINFORMED (PIN) auxin efflux carriers. PIN proteins have emerged in recent years from genetic studies in *Arabidopsis thaliana* as key regulators of a plethora of auxin-mediated developmental processes including axis formation in embryogenesis (Friml et al., 2003), postembryonic organogenesis (Okada et al., 1991; Benková et al., 2003; Reinhardt et al., 2003), root meristem maintenance (Friml et al. 2002b; Blilou et al., 2005), vascular tissue differentiation and regeneration (Scarpella et al., 2006; Sauer et al., 2006) and tropic growth (Luschnig et al., 1998; Friml et al., 2002a). PINs act as mediators of auxin efflux from cells (Petrasek et al., 2006) and show distinct polar subcellular localizations at the PMs of different cell types (Kleine-Vehn and Friml, 2008). Importantly, the polarity of PIN localization determines the direction of intercellular auxin flow (Wiśniewska et al., 2006) and thus provides the connection between cellular polarizing signals and the polarity of the whole tissue (Scarpella et al., 2006; Sauer et al., 2006).

An important factor for the polar delivery of PIN proteins to the plasma membrane is the endosomal regulator of the vesicle budding, GNOM, which encodes a guanine nucleotide exchange factor for adenosyl ribosylation factors (ARF GEF) (Shevell et al. 1994; Geldner et al. 2001, 2003). In *gnom* (also designated *emb30*) mutant embryos, the initial polarization of PIN1 to the basal side of internal cells is not established at the globular stage, leading to defective apical-basal axis formation, as also reflected by the failure of coordinated PIN polar localization at later stages (Kleine-Vehn et al. 2008a). Also, in postembryonic roots, GNOM function seems to be crucial for basal PIN targeting in particular, whereas the apical

delivery of PINs or the auxin influx carrier AUX1 (Bennett et al., 1996) is unaffected in *gnom* mutants (Kleine-Vehn et al. 2006, 2008a). Collectively, these studies demonstrate that apical cargoes utilize a targeting pathway that is molecularly distinct from that used by basally localized PIN proteins (Kleine-Vehn et al. 2006, 2008).

Apical and basal PIN targeting is achieved by the alternate use of distinct ARF-GEF dependent polar targeting pathways (Kleine-Vehn et al. 2008). Pharmacological inhibition of the ARF-GEF subclass by brefeldin A (BFA) interferes with the function of GNOM ARF GEF in endocytic recycling and results in the internalization of PIN cargoes from the basal plasma membrane domain into so called BFA compartments (Geldner et al., 2001; Kleine-Vehn et al., 2008). Prolonged pharmacological or genetic interference with GNOM function leads to the gradual appearance of the basal PIN cargo at the apical cell side. This process is independent of de novo protein synthesis and, therefore, hints at a dynamic PIN translocation between distinct polar plasma membrane domains via recycling endosomes, which was confirmed using photoswitchable version of PIN2 (Kleine-Vehn et al., 2008). The dynamic translocation of polar competent proteins from one functionally distinct plasma membrane to another one via recycling endosomes is termed transcytosis in animal cells (Knoblich, 2000). Thus, PIN proteins are capable to move between the apical and basal sides of cells in a manner similar to that of the transcytosis mechanism known in animal cells, illustrating that endocytic recycling and polar targeting in plants are linked. This mechanism is possibly also used for the rapid changes in PIN polarity in response to environmental (i.e., gravity) or developmental cues (Friml et al., 2002a; Friml et al., 2003; Benková et al., 2003). Such rearrangements reroute local auxin fluxes and contribute to regulation of various developmental processes (Friml et al., 2003).

Despite the prominent developmental importance of PIN polarity and its dynamic changes, the cellular and molecular mechanisms underlying PIN polar targeting and transcytosis are poorly characterized. Here, we examine the cellular and molecular requirements of these processes revealing their strong conservation in diverse *Arabidopsis* ecotypes and a previously unidentified level of complexity in regulations by actin cytoskeleton, microtubuli and various distinct ARF-GEF vesicle trafficking pathways.

Results

PIN polar targeting and transcytosis are strongly conserved among *Arabidopsis* ecotypes

Within a species, an ecotype is a genetically unique population that is adapted to its local environmental conditions. The resulting natural genetic diversifications can be generally used to estimate the evolutional pressure and thus the general importance of various processes in plant development. We used this approach to examine how conserved the processes of polar targeting, endocytic recycling and transcytosis of PIN proteins are.

We analyzed polar targeting of PIN1 and PIN2 in 30 selected ecotypes of *Arabidopsis* using the most frequently used ecotype *Columbia-0* as reference. Notably, apical and basal PIN targeting as visualized in the root meristem was largely unchanged. The basal PIN1 localization in stele and endodermis cells as well as apical and basal PIN2 localisation in epidermis and cortex cells, respectively, was not visibly altered in all 30 analysed ecotypes (Supl. Figure 1; Figure 1 A-B; D; F-G; I; K-L; N; P-Q; S).

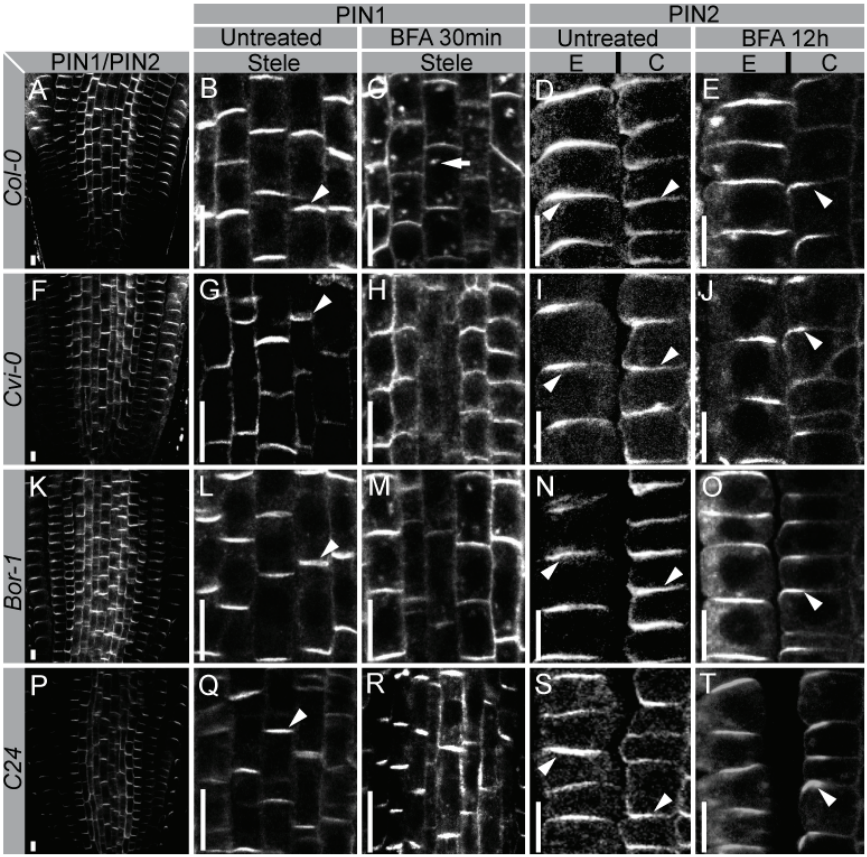
To compare endocytic recycling events of PIN proteins in different ecotypes, we made use of brefeldin A (BFA), a well known inhibitor of a subclass of ARF-GEF regulators of vesicle trafficking. One of the prominent molecular targets of BFA is GNOM ARF GEF (Geldner et al., 2003) that mediates PIN recycling from endosomes to the basal side of the cell (Kleine-Vehn et al., 2008). BFA treatment, thus, inhibits PIN recycling resulting in internalization of basal PIN cargoes into so called BFA compartments that are aggregations of various endocytic compartments (Geldner et al., 2003). Rapid appearance of PIN proteins in BFA compartments revealed the BFA-sensitive constitutive cycling of PIN proteins in all 30 analysed ecotypes (Supl. Figure 1), with some quantitative differences observed in about 10% of the analysed ecotypes (Supl. Figure 1). For example, *Bor-1*, *C24*, and *Cvi-0* displayed reduced intracellular accumulation of PIN1 after 30 minutes of BFA treatments (Figure 1 C; H; M; R) but normal response after 90 minutes of BFA treatment as compared to *Col-0* seedlings (not shown). This indicates either subtle differences in rate of PIN cycling or differences in the BFA-sensitivity of these ecotypes. However, the occurrence of endocytic cycling in all studied ecotypes and the relatively low degree of variation indicates the general importance of PIN cycling for the *Arabidopsis* development.

The transcytosis of PIN proteins between basal and apical cell side

can be induced by prolonged BFA treatment. Prolonged inhibition of basal PIN targeting under such conditions leads to the recruitment of PIN proteins to less BFA-sensitive apical trafficking pathway, resulting in reduced occurrence of basally localized PIN proteins in BFA compartments and their subsequent translocation to the apical cell side (Kleine-Vehn et al., 2008). Such BFA-induced basal-to-apical translocation of PIN1 in the stele (not shown) and of PIN2 in the cortex (Supl. Figure 1; Figure 1 E; J; O; T) was detected in all 30 ecotypes, suggesting operational transcytosis pathway.

Collectively, these results illustrate that polar PIN targeting, recycling and transcytosis are operational and to a high degree conserved in the diverse *Arabidopsis* ecotypes. These findings indicate that these processes are under constant evolutionary pressure and are therefore generally required for *Arabidopsis* development in a wide variety of environmental conditions.

Figure 1. Conserved polar PIN targeting and transcytosis in *Arabidopsis* ecotypes.
(A-T) PIN1 and/or PIN2 localization is shown in Col-0 (A-E), Cvi-0 (F-J), Bor-1 (K-O); and C24 (P-T) root cells. PIN1 and PIN2 double staining in root tips is presented in (A, F, K, P). Representative pictures of PIN1 localization in untreated (B, G, L, Q) and BFA [50μM for 30 min] (C, H, M, R) incubated seedlings are presented, illustrating slight changes in BFA response in Cvi-0, Bor-1 and C24 compared to Col-0. Apical/basal polarity is largely unchanged in the analyzed ecotypes as indicated by apical and basal PIN2 localization in untreated epidermis and cortex cells, respectively (D, I, N, S). BFA [50μM for 12h] induced transcytosis of basal cargoes such as PIN2 in cortex cells was not visibly altered in the analysed ecotypes (E, J, O, T).
Arrows depict PIN proteins in the BFA compartments and arrowheads PIN polarity. Abbreviations: E, epidermis; C, cortex. Scale bars, 10 μm.



Role of actin cytoskeleton in apical and basal PIN targeting

The maintenance of genetic determinants of PIN polar targeting, endocytic recycling and transcytosis throughout *Arabidopsis* ecotypes further demonstrates the important role of these processes in plant development. Nonetheless, the requirements of different cellular processes for dynamic PIN polar localization are still to a large extent unclear. In particular, the role of the cytoskeleton has not been analyzed in detail. The cytoskeleton determines cellular shape and, not least, provides guidance for vesicle trafficking in all eukaryotic cells.

The actin cytoskeleton is considered to provide primary guidance for vesicle trafficking in interphase plant cells and has been shown to be important for both auxin transport and PIN endocytic recycling (Dhonukshe et al., 2008; Geldner et al., 2001). On the other hand, apical targeting of AUX1 auxin influx carrier appears to be more sensitive to actin interference as compared to basal PIN1 targeting (Kleine-Vehn et al., 2006). This indicates more general differences in the role of actin cytoskeleton in apical versus basal trafficking. We initially used PIN2 as a marker for both apical and basal polar targeting because PIN2 displays apical (upper cell side) occurrence in root epidermal cells but preferential basal (lower cell side) in younger (close to root apex) adjacent cortex cells (Wisniewska et al., 2006). Notably, as cortex cells differentiate and move out of the meristem, a basal-to-apical polarity shift occurs resulting in apical PIN2 localization in older cortex cells (Figure 2A). Latrunculin B-induced actin depolymerisation strongly affected PIN2 localisation in epidermis and older cortex cells, whereas the effect on younger cortex cells was less pronounced (Figure 2B). This result indicates that, similar to apical AUX1 targeting, apical PIN2 targeting has stricter requirements for intact actin cytoskeleton than the basal targeting. On the other hand, when the actin requirements for apical versus basal PIN targeting was tested in the same cell type, using PIN2:PIN1-HA lines where PIN1 is targeted to the basal plasma membrane in root epidermal cells (Wisniewska et al., 2006), both apical PIN2 or basal PIN1 were comparably sensitive to actin depolymerisation (Figure 2C-D).

These experiments indicate that polar cargoes designated for the apical cell side have a stricter requirement for an intact actin cytoskeleton, however, both apical and basal PIN targeting clearly is facilitated along actin filaments.

Role of microtubules in apical and basal polar PIN targeting

In animal cells, vesicle transport along both actin filaments and microtubules has been illustrated and linked to various developmental processes. Notably, microtubules are mainly involved in perinuclear and actin filaments in cortical vesicle transport in animal interphase cells. However, both actin and micrtotubule cytoskeleton have been linked to the maintenance and establishment of animal cell polarity (reviewed in Goode et al., 2000).

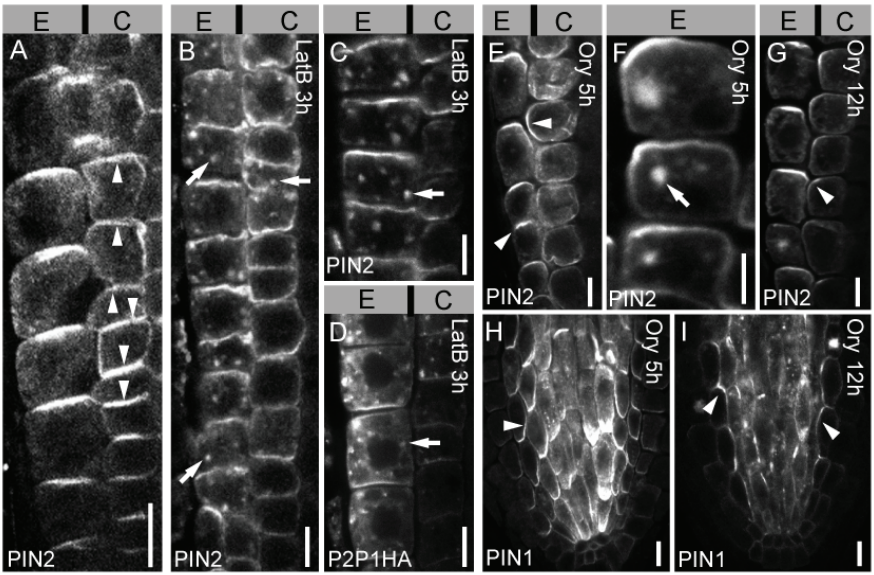
In plants, the role of the microtubule cytoskeleton for cell polarity has been implied (Boutte et al., 2006) but remains widely unknown. The rigid plant cell wall represents an additional determinant of their cell shape. The

synthesis of cell wall components have been linked to cortical microtubule (Paradez et al., 2006). Moreover, microtubules are instrumental for both plant and animal vesicle transport during cytokinesis (Van Damme and Geelen, 2008), however, their input to vesicle transport in interphase cells is ill-defined in plants.

We used the microtubule de-polymerizing agent oryzalin to examine the potential contribution of microtubules for apical and basal polar targeting in plant cells. Oryzalin treatments for 5 hours interfered visibly with basal PIN2 targeting in young cortex cells and with PIN1 targeting in stele, resulting in a reduced polar distribution (Figure 2E; H). In contrast, PIN2 occurrence at the apical cell side in epidermis cells was largely unaffected (Figure 2E). These findings indicate that intact microtubules are required preferentially for basal in comparison to apical PIN targeting. In contrast, we also observed weak PIN2 accumulation following microtubule depolymerization in epidermis cells (Figure 2F), suggesting that PIN2 intracellular trafficking, but not apical occurrence *per se* was compromised. In agreement with these findings, prolonged oryzalin treatments led to a preferential ectopic apical PIN2 occurrence in cortex cells (Figure 2G). Similarly, PIN1 in stele showed apical and lateral mislocalization after prolonged oryzalin treatments (Figure 2I).

Together these findings demonstrate that intact microtubules are required for polar trafficking in plant cells and identify a previously unidentified function in basal polar targeting.

Figure 2. Cytoskeletal requirements for polar PIN targeting.
(A-D) PIN2 (A-C) and PIN2:PIN1-HA (D) localization is shown in untreated (A) epidermis and cortex cells or after latrunculin B [20µM for 3h] treatment (B-D), revealing higher sensitivity of apically localized PIN2 in epidermal cells, however, the basal cargo PIN1 also showed comparable sensitivity to actin depolymerization in epidermal cells. Arrowheads in (A) indicate endogenous basal to apical polarity shift of PIN2 in upper cortex cells.
(E-I) PIN2 in cortex (E-G) and PIN1 localisation in stele (H-I) display oryzalin [20µM for 5h] (E-F; H) / [20µM for 12h] (G; I) senstitive polar targeting, suggesting microtubuli requirements for basal PIN targeting.
Arrows depict intracellular accumulation of PIN proteins and arrowheads PIN polarity. Abbreviations: E, epidermis; C, cortex. Scale bars, 10 µm.



Sequence-based and cell type determinants of ARF-GEF dependent transcytosis

The role of actin in polar targeting as well as previous studies (Wisniewska et al., 2006; Sauer et al., 2006) suggests that there are cell type- and cargo-specific influences on the PIN polar delivery. However, the cell type- and cargo-specific differences in dynamic PIN polarity changes, such as those related to ARF GEF-dependent transcytosis have not been addressed.

To obtain greater insight into this issue, we analyzed the cargo and cellular requirement of ARF-GEF dependent processes that dynamically guide PIN proteins to different polar destinations at the plasma membrane. We used BFA to stimulate ARF-GEF dependent PIN translocation from the basal to the apical cell side via recycling endosomes (Kleine-Vehn et al., 2008). Notably, the BFA induced basal-to-apical shift of PIN2 in young cortex cells was faster compared to the same translocation of PIN1 in the stele (Kleine-Vehn et al., 2008). Moreover, PIN2 proteins also undergo an intrinsic basal-to-apical shift in untreated upper cortex cells (Figure 2A), together suggesting a cell type influence on BFA-induced PIN polarity

changes. To specifically address the influence of cell type or PIN sequence for this targeting event in cortex cells, we ectopically expressed PIN1 sequences in the PIN2 expression domain. PIN2:PIN1-GFP3 shows apical localization in epidermis and basal localization in lower cortex cells, resembling the endogenous polar distribution of PIN2 (Wisniewska et al., 2006). Accordingly, BFA induced basal to apical shift of PIN1-GFP3 in lower cortex cells was comparable to PIN2 (Kleine-Vehn et al., 2008; Figure 3A-C). This result indicates that it is the cell type, more than sequence-specific determinants, that influences the BFA-sensitivity of the ARF-GEF dependent PIN transcytosis.

Furthermore, we found that some cell files in the stele after prolonged BFA incubation showed a basal-to-lateral PIN1 polarity switch in preference to a basal-to-apical shift (Figure 3D). Lateral PIN1 targeting in the same cells was also observed in partial loss of function mutants of *gnom*^{R5} (Figure 3F). Interestingly, it was the cell files adjacent to the protophloem cells that showed this ARF-GEF-related basal-to-lateral PIN1 polarity alteration (Figure 3E). These unexpected observations suggest that cell type not only determines the rate of BFA-induced PIN transcytosis but also influences the plasma membrane polarity domains, between which this transcytosis occurs.

However, beside the cell type determinants also the sequence of the polar cargo seems to influence the rate of BFA-induced transcytosis, because PIN1:HA (as compared to PIN2) showed a reduced basal-to-apical shift and more pronounced localization in BFA compartments even after long term BFA treatments when ectopically expressed in cortex cells (Figure 3G-I). Moreover, the ARF GEF-dependent transcytosis appears to be specific for polar PIN cargoes, because other polar cargoes such as the auxin influx carrier AUX1 did not get recruited to the apical cell side after prolonged BFA-dependent inhibition of the subclass of ARF-GEFs (Figure 3J-K). These findings confirm previous observations that auxin influx and efflux carrier trafficking is molecularly distinct (Damashiri et al., 2006; Kleine-Vehn et al., 2006) and illustrate that auxin influx and efflux carrier localizations can be independently regulated. Moreover, such a potentially exclusive PIN targeting pathway for PIN distribution in general and for PIN polarity alterations in particular could demonstrate the fundamental importance of PIN auxin efflux carrier targeting for plant development.

Here, we illustrate that, similar to the establishment of PIN polarity (Wisniewska et al., 2006), the ARF-GEF dependent re-arrangements of polar PIN distribution depends on both the cell type and the cargo itself.

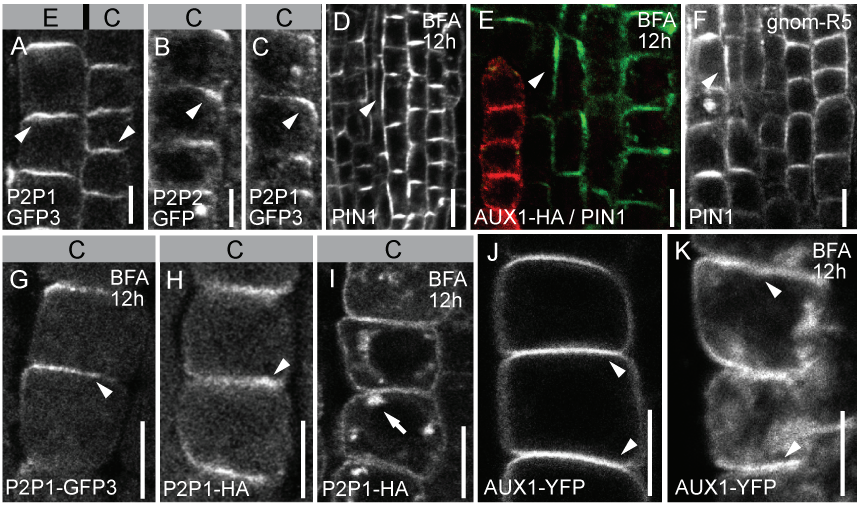


Figure 3. Cell type and PIN sequence determinants for polar PIN transcytosis.
(A-C) Ectopic PIN1-GFP3 expression under the PIN2 promotor (A,C) leads to apical and basal PIN1 distribution in epidermis and cortex cells (A), respectively. PIN2-GFP (B) and PIN1-GFP3 (C) showed comparable sensitivity to BFA [50µM for 3 hours] induced basal-to-apical transcytosis in cortex cells.
(D-E) long term BFA treatments [50µM for 12h] shows preferentially basal-to-apical polarity shift of PIN1, however, cell file dependent basal-to-lateral shift was detectable (D). Basal-to-lateral shift of PIN1 (green) was most readily seen adjacent to the protophloem cells (E) marked by AUX1-HA (red).
(F) PIN1 localization in *gnom*^{R5} is presented, showing basal-to-lateral polarity shift reminiscent to BFA treatments.
(G-H) long term BFA [50µM for 12h] treatments lead to a pronounced PIN1-GFP3 apicalization in cortex cells (G). Basal PIN cargo PIN1-HA in cortex (H) showed reduced basal to apical shift and persisted in BFA compartments (I).
(J-K) AUX1-YFP localization in untreated (J) and BFA [50µM for 12h] incubated root epidermal cells, displaying BFA insensitive AUX1

localisation at the apical and basal cell sides.
Arrows depict intracellular accumulation of PIN proteins and arrowheads PIN polarity. Abbreviations: E, epidermis; C, cortex. Scale bars, 10 µm.

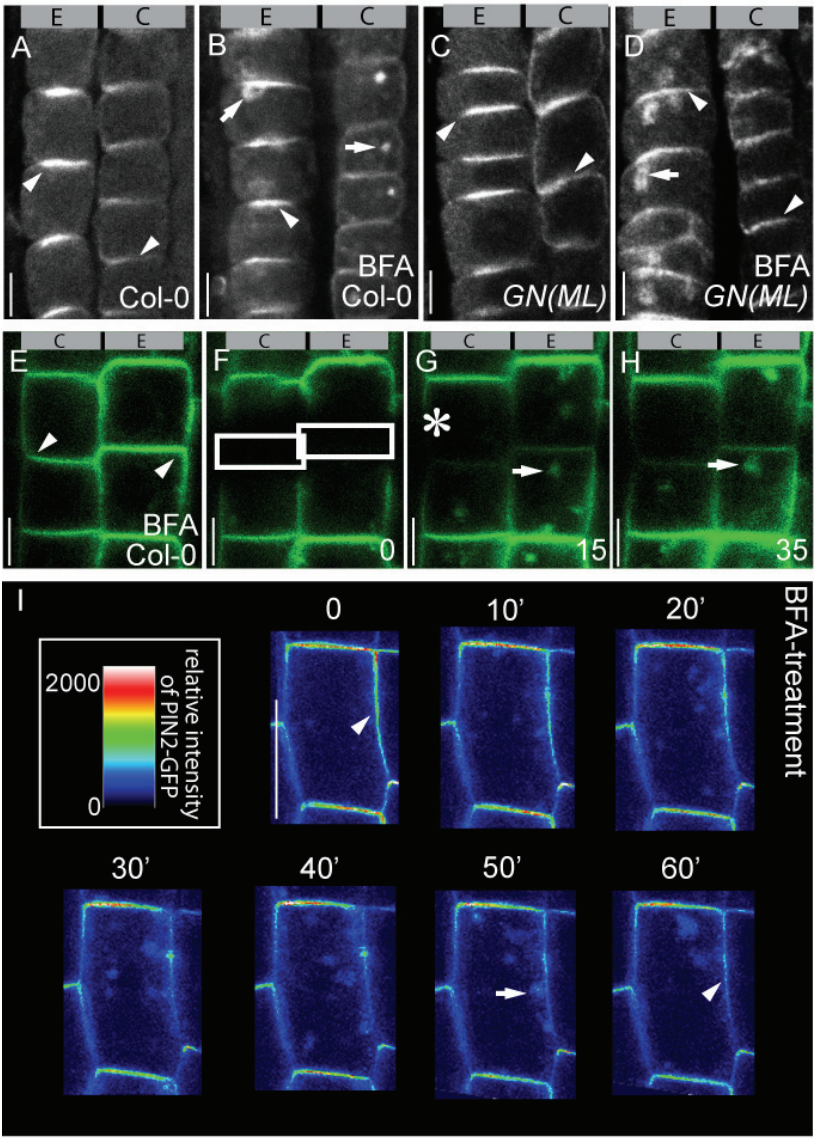
GNOM ARF-GEF independent trafficking of PIN proteins

It is possible that the cell type-specific influence on BFA-induced transcytosis is related to the different activity of distinct trafficking pathways. Therefore, we addressed the potential contribution of different ARF-GEF dependent pathways in polar PIN targeting. Our previous work suggested that the ARF-GEF GNOM regulates basal PIN targeting but not apical PIN targeting (Kleine-Vehn et al., 2008). We addressed this assumption directly by the use of an engineered BFA-resistant version of $GNOM^{ML}$ that resulted in BFA-insensitive targeting of PIN2 to the basal cell side in lower cortex cells (Figure 4A-D). In contrast, PIN2 still accumulated in BFA compartments in epidermal cells, however, remained polarly localized at the apical cell side in both wild type and $GNOM^{ML}$ seedlings (Kleine-Vehn et al., 2008; Figure 4B; D). These findings are consistent with the model that basal PIN2 targeting is GNOM-dependent, intracellular PIN2 targeting in epidermal cells at least to some degree is facilitated by a GNOM-independent but BFA-sensitive pathway, and the PIN2 occurrence at the apical cells side is largely insensitive to BFA treatment. Next, we addressed in detail the contribution of plasma membrane-derived PIN2 proteins to its intracellular accumulation in BFA compartments. Notably, apical PIN2 protein pool did not primarily contribute to the formation of BFA dependent intracellular PIN2 accumulation, because the removal of apical PIN2-GFP by photo-bleaching did not interfere with the event of intracellular PIN2-GFP accumulation in response to BFA treatment (Figure 4E-H). In contrast, photobleaching of the basal PIN2-GFP pool in young cortex cells largely compromised the intracellular accumulation after BFA treatment (Figure 4E-H). This live cell imaging study confirms our previous observations that BFA leads to the accumulation of basal but not apical PIN2 proteins (Kleine-Vehn et al., 2008). Next we addressed the origin of the BFA induced intracellular PIN2 accumulation in epidermis cells. Time lapse imaging revealed that lateral, but not apical, PIN2-GFP signal rapidly decreased in response to BFA treatments (Figure 4I). These findings indicate that the lateral PIN2 protein pool represents a main source for the BFA-induced intracellular accumulation of PIN2 in epidermal cells.

Here we illustrate that GNOM is a major regulator of basal PIN targeting while additional BFA insensitive and sensitive pathways appear to

regulate apical and lateral PIN targeting, respectively. Such diversity in ARF GEF dependent trafficking pathways might provide a basis for the observed cell type- and cargo-specific differences in polar targeting and transcytosis.

Figure 4. BFA sensitive but GNOM independent PIN targeting.
(A-D) PIN2 localization in untreated (A, C) and BFA [50μM for 1h] treated wild-type (A, B) and engineered BFA resistant version of $GNOM^{ML}$ (C, D), displaying GNOM independent intracellular PIN2 targeting in epidermis cells.
(E-H) apical and basal PIN2 proteins at the plasma membrane was bleached in epidermis and cortex cells, respectively (F) and the subsequent BFA [50μM] induced accumulation was monitored after 15 (G) and 35 (H) minutes, indicating that PIN2 accumulation in BFA compartments is largely independent of apical PIN2 protein pool in epidermal cells.
(I) false colour code was used to monitor PIN2-GFP intensity at the plasma membrane in response to BFA [50μM for up to 60min] application. Lateral PIN2 localization was sensitive to BFA treatment and contributes to PIN2 accumulation in BFA compartments. In contrast, apical PIN2 localization was largely resistant to the BFA treatment.
Arrows depict intracellular accumulation of PIN proteins and arrowheads PIN polarity. Abbreviations: E, epidermis; C, cortex. Scale bars, 10 μm.


E
C
A
Col-0
B
BFA
Col-0
C
GN(ML)
D
BFA
GN(ML)
E
BFA
Col-0
F
0
G
15
H
35
I
2000
0
relative intensity
of PIN2-GFP
0
10'
20'
BFA-treatment
30'
40'
50'
60'

ARF/ARF-GEF dependent pathways for apical PIN targeting and transcytosis

It is tempting to speculate that GNOM-independent, but BFA-insensitive and -sensitive pathways indicate the involvement of distinct BFA-resistant and -sensitive ARF-GEFs in polar PIN targeting. We tested this hypothesis by the genetic interference directly with ARF pathways. Notably, the conditional overexpression of a locked GTP-bound version of $ARF1^{QL}$ resulted in intracellular PIN2 accumulation (Figure 5A-B) indicating that indeed the ARF pathway is also involved in apical PIN targeting. Moreover, BFA-induced polar PIN transcytosis was compromised in ARF^{QL} lines (Figure 5C-D). These findings suggest that, in addition to GNOM ARF GEF involvement, ARF-GEF/ARF pathways are instrumental for polar PIN targeting. These results are in agreement with BFA-resistant apical PIN targeting (Kleine-Vehn et al., 2008), and strongly suggest that (a) BFA resistant ARF-GEF(s) is (are) required for apical PIN targeting as well as for transcytosis.

The GNOM homolog GNOM LIKE1 (GNL1) has been shown to be BFA resistant and to localize to, and act primarily at Golgi stacks, regulating COPI-coated vesicle formation (Richter et al., 2007). Moreover, GNL1 was proposed to be also required for internalization from the plasma membrane in the presence of BFA (Teh and Moore, 2007). We tested whether the BFA-resistant GNOM LIKE1 might additionally contribute to apical PIN transcytosis. In both, the engineered BFA-sensitive version of $GNL1^{LM}$ and the *gnl1-3* mutants, long term BFA treatment still induced the basal-to-apical PIN2 polarity shift in young cortex cells (Figure 5E), suggesting that the BFA-insensitive apical PIN delivery is GNL1 independent.

These findings collectively illustrate the complexity of ARF-GEF dependent PIN targeting to the different cell sides. Basal PIN targeting largely depends on the ARF-GEF GNOM, while lateral and apical PIN2 targeting is apparently independent of GNOM. Our data illustrates that apical targeting is also dependent on the ARF pathway and, hence, implies the involvement of (a) BFA resistant ARF-GEF(s) in this process. However, the BFA-resistant GNL1 does not seem to be strictly required for the apical PIN targeting or basal-to-apical transcytosis.

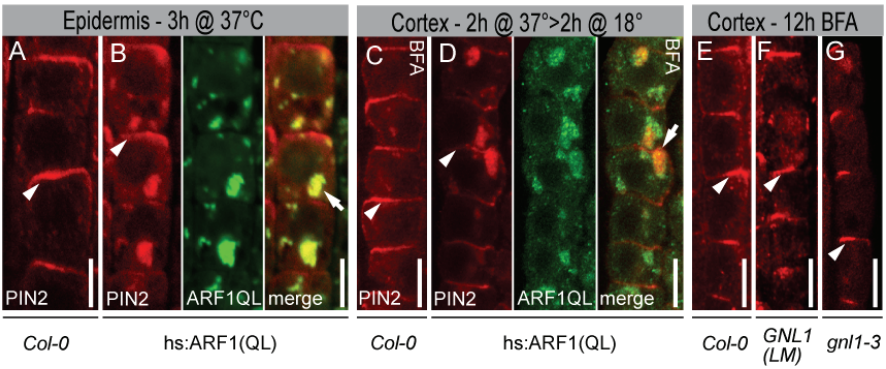


Figure 5. ARF/ARF-GEF dependency for apical PIN transcytosis.
(A-B) apical PIN2 (red) localisation in epidermis cells (A) is compromised after heat shock induction of a GTP locked version of $ARF1^{QL}$ (green), leading to ARF1/PIN2 intracellular accumulation and reduced PIN2 labelling at the plasma membrane.
(C-D) BFA treatments lead to the basal-to-apical transcytosis of PIN2 in cortex cells, while $ARF1^{QL}$ (green) expression reduces PIN2 (red) translocation to the apical cell side (D).
(E-G) long term BFA [50µM for 12h] incubations resulted in apical PIN2 localization in wild type (E), engineered BFA sensitive $GNL1^{LM}$, and *gnl1-3* mutants, suggesting GNL1 independent PIN transcytosis to the apical cell side.
Arrows depict intracellular accumulation of PIN proteins and arrowheads PIN polarity. Abbreviations: E, epidermis; C, cortex. Scale bars, 10 µm.

Discussion

The polar distribution of PIN proteins correlates with, and determines the polar flow of the plant hormone auxin during numerous processes in plant development (Friml et al., 2003; Leyser, 2005). In this work we addressed some unclarified issues in cellular and genetic requirements for PIN polar targeting and transcytosis-mediated polarity changes.

The remarkable conservation of PIN polar targeting, BFA-sensitive endocytic recycling and BFA-induced transcytosis in 30 selected different *Arabidopsis* ecotypes shows that the mechanisms underlying the control of PIN polar targeting and PIN polarity switches are under evolutionary pressure, reflecting their overall importance for regulating plant

development.

Our studies also revealed previously unknown aspects of cytoskeletal requirements for apical and basal PIN targeting. Despite the evidence that apical targeting is more sensitive to actin depolymerisation compared to basal targeting, both apical and basal polar targeting depend on the actin cytoskeleton. This corresponds well to the situation in animal epithelial cells, where distribution of polar cargos has been also suggested to be differentially sensitive to actin manipulations (Rosin-Arbesfeld et al., 2001; Tamma et al., 2005). Apart from the actin cytoskeleton, microtubules also appear to be important for polar PIN localization. So far, conclusive data were available only for role of microtubules in the PIN targeting to the forming cell plate in dividing cells (Geldner et al., 2001). Here we show that, in particular, the basal PIN targeting was disturbed after microtubule depolymerization, showing, in the most severe cases, even a basal-to-apical polarity shift of PIN localization. In the case of PIN targeting, prolonged microtubule depolymerization clearly interferes with vesicle transport in plant interphase cells, however, it remains to be seen whether this reflects direct vesicle transport events along microtubules or, alternatively, reflects secondary effects such as cell wall/shape alterations that lead to potential loss of basal polarity.

Plant cells posses the remarkable ability to re-determine the polarity of already predefined tissues, contributing substantially to their flexible postembryonic development. The polarity of PIN localization can be rapidly rearranged in response to environmental or developmental cues (Friml et al., 2002a; Friml et al., 2003; Benková et al., 2003). Such rearrangements reroute local auxin fluxes and thus trigger various developmental processes and represent important cues for tissue polarity (Sauer et al., 2006). Despite the fundamental importance of PIN polarity rearrangements for plant development, the underlying mechanisms are to date surprisingly ill defined. Here, we illustrate the unexpected complexity of various ARF and ARF GEF-dependent polar trafficking pathways that underlie PIN polarity changes. The data revealed a strong cell type-dependent component of this type of polar targeting regulation. In some cases, the cell type appears to not only predetermine the rate of transcytosis but also the polar domains for ARF-GEF dependent PIN transcytosis. The most extreme example is the BFA-induced basal-to-lateral PIN1 translocation in cells neighbouring the protoploem as compared to basal-to-apical transcytosis in most other root cell types. Nonetheless, apart from cell type determinants, PIN recruitment to the BFA-insensitive apical targeting

pathway and subsequent apical transcytosis (Kleine-Vehn et al., 2008) depends on the PIN sequence itself. Moreover, BFA induced polarity alterations from the basal-to-apical cell side appear to be specific for polar PIN cargoes, because polar competent AUX1 could not be rerouted by prolonged BFA incubations. This finding illustrates distinct targeting pathways for auxin influx and efflux carriers, enabling independent regulation of auxin influx and efflux in individual cells (Kleine-Vehn et al., 2006).

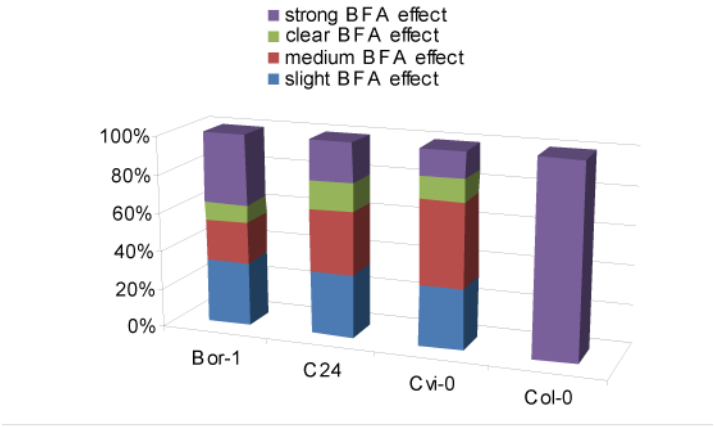
Collectively, our findings illustrate that distinct ARF/ARF-GEF dependent pathways are instrumental for PIN targeting to different cell sides. As shown also previously (Kleine-Vehn et al., 2008), we confirmed that basal PIN targeting is strictly dependent on the function of GNOM ARF-GEF. Furthermore, while apical PIN targeting appears to be regulated by another, BFA-insensitive ARF-GEF, lateral PIN2 occurrence is most likely mediated by a GNOM-independent, but BFA-sensitive ARF-GEF pathway. Our studies also show that the BFA-insensitive ARF-GEF GNL1 is not directly required for apical targeting or basal-to-apical transcytosis. Since the sequence predictions indicate that no other ARF GEF of the GBF clade would be BFA-insensitive (Geldner et al., 2003), these processes may be regulated by so far uncharacterised ARF GEF(s) of the BIG class.

The utilization of distinct ARF-GEF vesicle regulators for polar distribution to distinct plasma membrane domains appears to have a clear functional role in case of PIN proteins. The independent deposition of PIN proteins by distinct targeting pathways could enable rapid PIN polarity changes by alternative recruitment by these different pathways. PIN positioning to different sides of cells defines auxin efflux in a three dimensional manner at a level of individual cells and, thus, could substantially contribute to the creation of an auxin distribution map for defining pattern of plant development.

Ecotype	PIN polarity	BFA response [50µM] for 30 min	BFA response [50µM] for 12h
An-1	not changed	not changed	apicalization
Bay-0	not changed	not changed	apicalization
Bor-1	not changed	reduced	apicalization
Bor-4	not changed	reduced	apicalization
C24	not changed	reduced	apicalization
CIBC-17	not changed	not changed	apicalization
Col-0	not changed	not changed	apicalization
Cvi-0	not changed	reduced	apicalization
Eden-1	not changed	not changed	apicalization
Ei-2	not changed	not changed	apicalization
Ga-0	not changed	not changed	apicalization
Got-7	not changed	not changed	apicalization
Gu-0	not changed	not changed	apicalization
Gy-0	not changed	not changed	apicalization
Kas-X	not changed	not changed	apicalization
Kin-0	not changed	not changed	apicalization
Kz-9	not changed	not changed	apicalization
Ler-1	not changed	not changed	apicalization
LL-0	not changed	not changed	apicalization
Mr-0	not changed	not changed	apicalization
Mt-0	not changed	not changed	apicalization
Po-0	not changed	not changed	apicalization
Ren-1	not changed	not changed	apicalization
Se-0	not changed	not changed	apicalization
Sha	not changed	not changed	apicalization
Spr1-2	not changed	not changed	apicalization
Tsu-1	not changed	not changed	apicalization
Ull2-3	not changed	not changed	apicalization
Uod-1	not changed	not changed	apicalization
Ws-0	not changed	not changed	apicalization

S.Figure 1. PIN targeting in various *Arabidopsis* ecotypes.
Table summarizes immunolocalization studies on PIN polarity and transcytosis.
Apical/basal PIN targeting was largely unchanged among the diverse ecotypes, however, BFA [50µM for 30 min] sensitivity was altered in about

10% of the analyzed ecotypes. BFA induced basal-to-apical PIN transcytosis was detectable in all analyzed ecotypes.



S.Figure 2. PIN cycling in *Arabidopsis* ecotypes.
The chart depicts variable BFA [50µM for 30 min] sensitivity of PIN targeting in Bor-1, Cvi-0, and C24 compared to Col-0.

Experimental Procedures

Used materials

gnom^{R5} (Geldner et al., 2004); *GNOM*^{M696L} (Geldner et al., 2003); *PIN2:PIN2-GFP*, *PIN2:PIN1-HA*, and *PIN2:PIN1-GFP-3* (Wisniewska et al., 2006); hs:ARF1^{QL} (Xu and Scheres, 2005); *AUX1-HA* (Swarup et al., 2001); *AUX1-YFP* (Swarup et al., 2004); *GNL1*^{LM}*-YFP* in *SEC-GFP*, *GNL1-YFP* in *SEC-GFP*, *gnl1-3* (Teh and Moore, 2007) have been described previously.

Growth conditions

Plants were grown on soil or MS plates as described [Friml et al., 2002] under a 16-h light/8-h dark photoperiod at 21/18°C.

Drug applications and experimental conditions

Exogenous drugs were applied by incubation of 5-days-old seedlings in liquid or solid half-strength Murashige and Skoog (MS) medium supplemented with BFA (50 mM stock in dimethylsulfoxide [DMSO]) (50 µM), latrunculin B (20 mM stock in DMSO) (20 µM) or oryzalin (20 mM stock in DMSO) (20 µM). Control treatments contained an equivalent amount of solvent (DMSO).

For all treatments, markers, and mutant phenotype analyses, control experiments were done in the sister lines and a total number of at least 36 roots for each treatment were analyzed and representative images are presented. For all comparisons, independent experiments were done at least in triplicate with the same significant results. Data were statistically evaluated with Excel, 2003 (Microsoft).

Expression and immunolocalization analyses

Whole-mount immunofluorescence was performed as described [Friml et al., 2003]. Antibodies were diluted as follows: 1:2000 for rabbit anti-PIN1 [Paciorek et al., 2005]; 1:2000 for rabbit anti-PIN2 (generously provided by C. Luschnig); 1:500 rabbit anti-GFP (Molecular probes); mouse anti-GFP 1:800 (Roche) and 1:500 and 1:600 for FITC- and CY3-conjugated anti-mouse and anti-rabbit secondary antibodies (Dianova), respectively. GFP was visualized in 5% glycerol or in chambered cover glass (Nunc) submerged with solid MS media without fixation for live-cell imaging. For the photobleaching experiments 5 to 6 days old seedlings were mounted in

liquid media on slides or in chambered cover glass (Nunc) submerged with solid MS media. A region of interest was selected for scans using the Leica LCS confocal software 2004 (Leica) FRAP procedure as previously described [Kleine-Vehn et al., 2006]. GFP images before and after scans were collected (n=4) and representative images are presented. For confocal laser scanning microscopy, single scans were obtained with Leica TCS SP2 AOBS or Olympus FV10 ASW. Images were processed in Adobe Photoshop and Illustrator CS2 (Adobe Inc.).

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Chapter 5

Result part IV:

Differential Degradation of PIN2 Auxin Efflux Carrier by SNX1-Dependent Vacuolar Targeting

Differential degradation of PIN2 auxin efflux carrier by SNX1-dependent vacuolar targeting

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Summary
All eukaryotic cells present a specific set of plasma membrane proteins that modulate responses to internal and external cues. Among various mechanisms, their activity is also regulated by tightly controlled protein degradation. Here, we characterized lytic vacuole-dependent protein degradation in *Arabidopsis thaliana* by means of *in vivo* visualization of vacuolar targeting combined with quantitative protein analysis. We identified several cargos of this pathway, including PIN-FORMED (PIN) efflux carriers for the phytohormone auxin [1]. *In vivo* visualization of PIN2 turnover revealed its differential degradation in response to environmental signals such as gravity. In contrast to PIN delivery to the basal plasma membrane, which depends on the vesicle trafficking regulator ARF-GEF GNOM [2, 3], PIN sorting to the lytic vacuolar pathway requires additional Brefeldin A-sensitive ARF-GEF activity. Furthermore, we identified SORTING NEXIN1 (SNX1) [4] as an important component of this pathway and propose a PIN protein retrieval function from a late/pre vacuolar compartment for the retromer complex. Our data suggest that ARF GEF- and SNX1-dependent processes regulate PIN sorting to the vacuole in a counter directional manner and illustrate its instrumentalisation for fine tuning the auxin fluxes during plant development.

Introduction
Plants evolved a remarkable developmental plasticity to shape their individual growth according to environmental stress. The signal molecule auxin has been ultimately linked to the flexible plant expansion. The distribution of the phytohormone auxin depends largely on its directional

transport from cell to cell (Tanaka et al., 2006). Auxin efflux carriers of the PIN-FORMED (PIN) family show polar localisation that correlates with and determines the direction of auxin flow (Wisniewska et al., 2006; Petrasek et al., 2006). By the action of this delivery system, auxin accumulates in spatial and temporal patterns, which are read out to direct plant growth and development (Leyser, 2006; Kleine-Vehn and Friml, 2008). Throughout the plant's life, auxin contributes to developmental adaptation such as postembryonic organ formation, apical dominance, tissue regeneration and tropisms (Friml et al., 2002, 2003; Benkova et al., 2003, Sauer et al., 2006).

PIN auxin efflux carrier targeting displays a highly dynamic process with constitutive cycling of the PINs between the plasma membrane and (an) endosomal compartment(s). This process depends on ADP-ribosylation factor GTP-exchange factors (ARF-GEFs) proteins such as GNOM (also called EMB40), and is sensitive to vesicle trafficking inhibitors such as brefeldin A (Geldner et al., 2001, 2003; Friml et al., 2002b). GNOM appears to regulate PIN recycling strictly to the basal plasma membrane (Kleine-Vehn et al., 2008), while the putative retromer complex component SORTING NEXIN1 (SNX1) resides in intracellular structures distinct of GNOM and has been suggested to be involved in apical PIN2 but not basal PIN1 recycling (Jaillais et al., 2006). SNX1 genetically interact with other putative retromer complex members such as VACUOLAR PROTEIN SORTING29 (VPS29) (Jaillais et al., 2007), but in contrast to SNX1, the VPS29 has been proposed to be involved preferentially in basal PIN1 targeting and has been suggested to function downstream of GNOM (Jaillais et al., 2007). Nevertheless, the contribution of the retromer complex to the PIN recycling remained controversial (Jürgens and Geldner, 2007).

However, the overall function of the PIN constitutive cycling remains unclear, though it has been proposed to enable the rapid changes in PIN polarity in response to environmental stimuli (Friml et al., 2002b; Kleine-Vehn et al., 2008) or to control the levels of PIN proteins at the plasma membrane and thus the activity of auxin transport (Paciorek et al., 2005). The activity of the PIN-dependent auxin distribution network can be regulated at multiple levels, including transcription [7-9] polar subcellular localization [10, 11], endocytosis [12] and, not least, protein degradation [13-16]. In particular, the mechanisms that regulate PIN degradation are still widely elusive. However, after gravi-stimulation, proteasome-dependent variations in PIN2 localization and degradation at the upper and

lower sides of the root result in asymmetric distribution of PIN2, suggesting functional importance for posttranslational regulation of PIN stability for plant development (Abas et al., 2006).

The mechanisms underlying the degradation of plasma membrane proteins are to date not well understood in plants, hence, we examined the cellular and molecular requirements of this process with a special focus on PIN efflux carriers. We used a drug free assay to illustrate PIN degradation in lytic vacuoles and illustrate its importance for fine tuning auxin transport during plant development. In particular, we illustrate that recycling and vacuolar degradation are interdependent but molecularly distinct. Our data suggest that a partially BFA sensitive ARF-GEF promotes the PIN protein transition from endosomes to the PVC, while SNX1 can counteract this process by retrieving PINs from the PVC, which appears to have functional importance during temporal PIN degradation during gravitropic response. Hence, we propose that the SNX1/VPS29-dependent retromer complex regulates the homeostasis of PIN proteins, but is not directly required for its recycling events to the plasma membrane.

Results and Discussion

Auxin efflux carrier PIN2 displays dynamic turnover in the lytic vacuole

To visualize the targeting to the lytic vacuole for degradation, we initially used concanamycin A, a well established and specific inhibitor of vacuolar H-ATPases, to reduce acidification of lytic compartments and, thus, protein degradation [17]. Under our experimental conditions, concanamycin A treatments enabled us to visualize accumulation and, hence, trafficking of fluorescently tagged plasma membrane proteins to the lytic vacuole for degradation, namely of the auxin efflux carrier PIN2 (Figure 1A-B), brassinosteroid receptor BRI1 (Figure 1D-E) and the aquaporin PIP2 (Figure 1G-H).

Because concanamycin A might have unwanted side effects on protein trafficking [19], we made use of the fact that green fluorescent protein (GFP) and related proteins are far more stable in lytic vacuoles under dark conditions than in light due to conformational changes [18], in order to study plasma membrane protein degradation in a drug-independent manner. Indeed, dark treatment led to vacuole-like accumulation of the GFP signal in various transgenic lines, including PIN2-GFP, BRI1-GFP, and PIP2-GFP (Figure 1C; F; I).

GFP localization in dark-treated PIN2-GFP coincided with the vacuolar occurrence in transmission light images (Figure 1J), confirming that the diffuse GFP signal following dark treatment revealed vacuole-targeted proteins. Moreover, the endocytic dye FM4-64, which labels the tonoplast within 2 h of incubation [19], surrounded the diffuse GFP signal in PIN2-GFP-expressing seedlings following 2 h in the dark (Figure 1K). These findings confirm that GFP resides in the lumen of the tonoplast after dark treatment, thus illustrating the degradation of PIN2 in lytic vacuoles.

To roughly estimate the turnover of PIN2, we analyzed the earliest GFP accumulation in lytic vacuoles. A diffuse GFP occurrence in lytic vacuoles was already detectable after 1-2 h in the dark in two independent PIN2-GFP [14, 20] lines (Figure 1K; data not shown). In contrast, BRI1-GFP fusions [21, 22] displayed their earliest vacuolar signals within 4-6h (Figure 1F; data not shown), in agreement with previous half life estimations of BRI1-GFP [22]. To investigate the PIN2 protein stability independently of the light-to-dark transition, dexamethasone-inducible TA:PIN2-GFP lines were used [14]. After induction of the transgene and subsequent removal of dexamethasone, PIN2-GFP was rapidly depleted, further indicating a fast, proteolytic turnover of this protein (Suppl. Figure 1).

These data show that integral plasma membrane proteins, such as PIN2, BRI1, and PIP2 are targeted to the vacuole for degradation. Moreover, we illustrated the rapid turnover of the PIN2 protein, suggesting a tight posttranslational regulation during plant development.

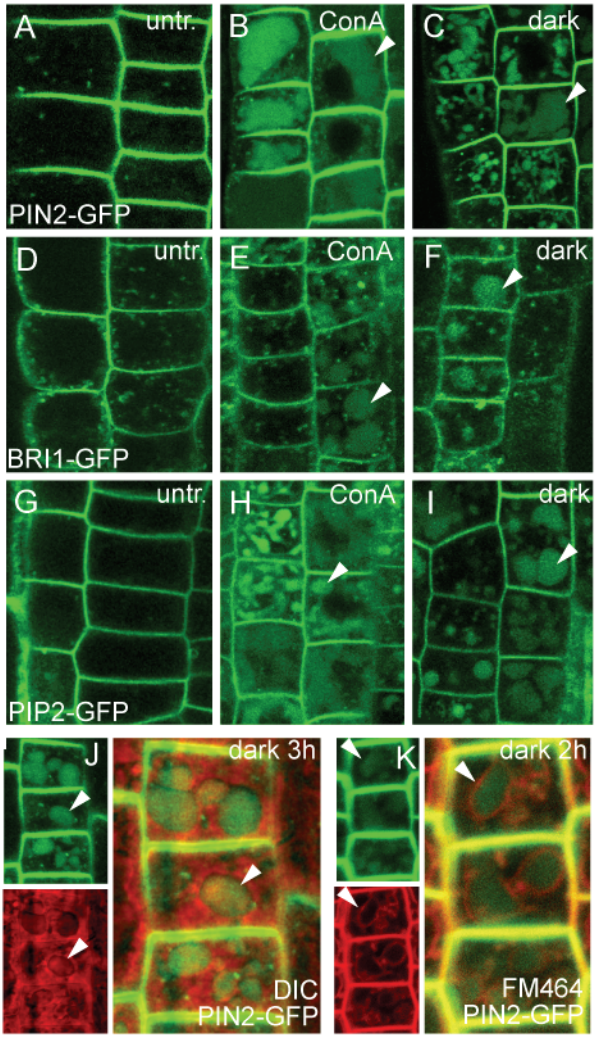


Figure 1. Visualization of trafficking of plasma membrane proteins to the lytic vacuole.
(A-C) PIN2-GFP localization at the plasma membrane and endocytic intracellular compartments in untreated epidermal root cells (A).

Appearance of GFP signal in lytic vacuoles in PIN2-GFP expressing seedlings after concanamycin A [1 μM/6 h] treatment (B) or incubation [6 h] in the dark (C).
(D-F) BRI1-GFP-expressing transgenic lines showing BRI1 localization at the plasma membrane and in intracellular structures (D). BRI1-GFP degradation in lytic vacuoles after concanamycin A (E), and dark (F).
(G-I) PIP2-GFP distribution in epidermis cells (G), after concanamycin A (H) and dark (I) treatments.
(J-K) Appearance of diffuse vacuolar GFP signal (in green) in PIN2-GFP expressing cells following dark treatment (2 h) were identified morphologically in transmission light image (in red) (J) or by endocytic uptake of FM4-64 (K) dye (in red) labelling the tonoplast around the diffuse GFP signal.
Arrowheads indicate vacuolar occurrence of GFP signals.

ARF-GEF-dependent trafficking of PIN2 to the lytic vacuole

To gain insights into the biological function of the PIN2 targeting to the lytic vacuole, we examined the cellular requirements for endocytic trafficking of PIN2. The PIN2-GFP targeting to the vacuole for degradation depends on actin cytoskeleton, because latrunculin B-induced depolymerization of actin inhibited GFP accumulation in lytic vacuoles and stabilized PIN2 as manifested by increased total levels of the PIN2 protein (Figure 2B and 2N), implying an actin-dependent vesicle transport for vacuolar PIN2 trafficking. Brefeldin A (BFA), an inhibitor of ARF-GEF-type vesicle transport regulators, restrains recycling to the plasma membrane and leads to PIN accumulation in aggregated endosomes [23] and also elevates PIN2 protein levels (Figure 2N) [14]. Notably, BFA reduced the PIN2 targeting to the lytic vacuole (Figure 2D), suggesting the involvement of an ARF-GEF. Moreover, BFA also inhibited the uptake of FM4-64 to the tonoplast membrane (Figure 2H) and stabilized BRI1 in endosomes [22], hinting at a general requirement for BFA-sensitive ARF GEF activity in the control of membrane trafficking from endosomes to the vacuole. The BFA-sensitive ARF-GEF GNOM is a major regulator of polar PIN recycling [2, 24]. However, reduced BFA concentrations sufficient to inhibit GNOM-dependent PIN recycling to the basal cell side [2] did not fully abolish endocytic PIN2-GFP or FM4-64 trafficking to the tonoplast (Figure 2C and G), which might indicate a GNOM-independent mode of action for BFA. To determine whether BFA affects the PIN2 trafficking to the vacuole independently from GNOM, we used an engineered BFA-resistant version

of GNOM [24]. The BFA-resistant *GNOM*^{ML} lines still showed BFA sensitivity for endocytic uptake of PIN2 and FM4-64 to the vacuole (Figure 2E and I), indicating a GNOM-independent vacuolar trafficking and involvement of an additional ARF GEF. This involvement of ARF and ARF-GEF activity in the PIN2 vacuolar targeting has been further substantiated by conditional overexpression of a constitutively active version of an ARF-GEF substrate ARF1 [20] that strongly interfered with the endocytic FM4-64 trafficking to the tonoplast (Figure 2J).

Thus, while polar recycling of PIN proteins to the basal plasma membrane depends on the GNOM function, endocytic translocation of plasma membrane components, such as PIN2, to the vacuole is GNOM independent and most probably utilizes another BFA-sensitive ARF-GEF activity. Importantly, these findings indicate that, following internalization, the PIN recycling to the plasma membrane and the alternative posting to the vacuole for degradation are molecularly distinct by ARF-GEF utilization.

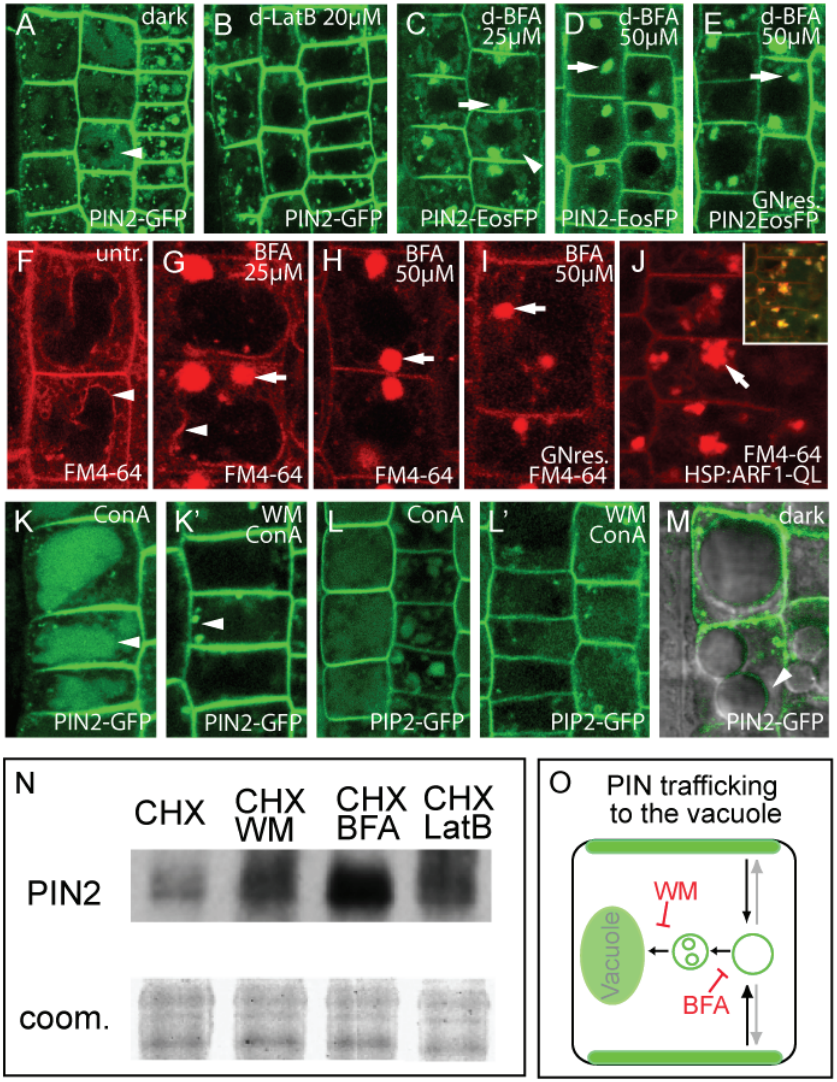


Figure 2. Cellular and molecular requirements of the PIN trafficking to lytic vacuoles.

(A-B) Latrunculin B (LatB) [30 µM] treatment (B) as compared to control (A) reveals actin dependent trafficking of the PIN2-GFP to the vacuole as visualized by dark treatment (2 h).
(C-E) Dark treatments (2 h) illustrates reduction of PIN2-EosFP in vacuolar targeting at [25 µM] BFA (C) and a complete block at [50 µM] BFA (D). In the transgene carrying engineered BFA-resistant version of GNOM ARF GEF ($GNOM^{ML}$), the vacuolar trafficking of PIN2-EosFP is still BFA-sensitive (E).
(F-J) ARF GEF-dependent FM4-64 uptake (2 h) to the tonoplast (indicated by arrowheads) of untreated (F), [25 µM] BFA (G), [50 µM] BFA (H), [50 µM] BFA, in the BFA-resistant version of $GNOM^{ML}$ (I), and after heat-shock induction [37°C; 2 h] of the constitutively active $ARF1^{QL}$ (J). Inset in (J) shows FM4-64 and $ARF1^{QL}$-YFP colocalization.
(K-L) Concanamycin A [1 µM, 6 h]-visualized trafficking of PIN2-GFP (K) and PIP2-GFP (L) to vacuoles was inhibited by wortmannin [15 µM, 6 h] (K' and L') treatment.
(M) Occasional PIN2 localization at the tonoplast after wortmannin [15 µM, 3 h in the dark] treatment.
(N) PIN2 protein-stabilizing effect of wortmannin (WM) Brefeldin A (BFA), and latrunculin B (LatB) by quantitative western analysis. Concomitant drug treatment with protein biosynthesis inhibitor cycloheximide (CHX) was done for inhibition of the PIN2 secretion.
(O) Schematic representation of the ARF-GEF (BFA) and PI3K (WM)-sensitive sorting events of PIN2 to the lytic vacuole for degradation.
Arrows mark BFA dependent accumulation and arrowheads the PIN2 occurrence in vacuoles or endocytic mistargeting

Vacuolar trafficking of PIN2 depends on PI3K activity

Progress has been made in the elucidation of the cellular machinery, by which cargo is delivered to the lysosome/vacuole in animal, yeast, and plant cells [25]. In plants, wortmannin, an inhibitor of phosphatidyl-inositol-3-kinase (PI3K), affects recycling of vacuolar sorting receptors between the prevacuolar compartment (PVC) and the trans Golgi network (TGN) and thus alters the PVC identity [26, 27]. In accordance, wortmannin severely affects the PVC morphology of treated plant cells [28].

Notably, wortmannin has been shown to interfere with apical PIN2 localization in epidermis, but not with basal PIN1 targeting in stele cells, indicating that wortmannin interferes with apical but not basal PIN trafficking [4]. However, ectopic expression of basal PIN1 cargo in root

epidermis cells resulted in comparable wortmannin sensitivity (data not shown). Similarly, we observed wortmannin sensitive PIN1 targeting in stele after prolonged wortmannin treatments (data not shown). Hence, differential sensitivity to wortmannin could be explained by cell type dependency or drug uptake. This finding indicates that wortmannin treatment does not discriminate between apical and basal polar cargos in plant cells.

Although, wortmannin has been suggested to interfere with PIN2 recycling events to the plasma membrane [4]; the underlying mechanisms remain unclear. We show that wortmannin abolishes vacuolar trafficking of PIN2-GFP (Figure 2K and K') as well as of additional plasma membrane proteins, such as PIP2-GFP (Figure 2L and L'/data not shown). Consistent with the observed effects of wortmannin on vacuolar targeting, the drug also increased the total PIN2 protein levels in membrane protein preparations (Figure 2N). From these findings, we concluded that posttranslational regulation of plasma membrane proteins, such as PIN2, involves PI3K signaling as a regulator, of protein translocation to the lytic vacuole.

As in tobacco cell cultures, wortmannin has been shown to reduce the endocytic uptake of FM1-43 [29], hence, we analysed if the observed effect on the PIN2 degradation was potentially a consequence of an overall reduced endocytosis in *Arabidopsis* root cells. Under our experimental conditions, early internalization of FM4-64 from the plasma membrane into the intracellular compartments was not severely altered (Suppl. Figure 2A-B and 2E-F). Also, wortmannin treatment did not abolish BFA-dependent intracellular accumulation of FM4-64 (Suppl. Figure 2D and 2H), clearly demonstrating the ongoing endocytosis in *Arabidopsis* epidermal root cells. In contrast, the endocytic targeting of FM4-64 to the tonoplast was reduced after wortmannin treatment (Suppl. Figure 2C and 2G). Thus, wortmannin affects the endocytic targeting of plasma membrane proteins to the vacuole by inhibiting intracellular sorting events rather than endocytosis at the plasma membrane. In accordance, in some cases we observed mistargeting of PIN2-GFP to the tonoplast membrane after wortmannin treatment (Figure 2M), suggesting sorting/invagination defects at the multi-vesicular body/pre-vacuolar compartment [28].

Taken together, the effect of wortmannin on the intracellular compartments disrupting vacuolar sorting of plasma membrane proteins, such as PIN2, strongly indicates that the PI3K activity is required for the regulation of vacuolar degradation of PIN2.

SNX1 localization and conditional mutant phenotypes suggest their involvement in vacuolar targeting

SNX1 is a potential downstream effector of PI3K because it bears a phosphatidylinositol binding PX domain [30] and colocalizes with a marker for phosphatidyinositol-3-phosphate (PI3P) enriched membrane subdomains (Figure 3A). Yeast orthologs of SNX1 are retromer complex components required for vacuolar receptor retrieval from the PVC to the TGN in a clathrin- and COP-independent manner [31]. In plants, putative retromer components localize to the PVC and might interact with vacuolar sorting receptors [32]. Moreover, SNX1 has been shown to co-localize and genetically interact with the putative plant retromer component VPS29 [33]. In agreement with these findings, we found another putative plant retromer component [32], VPS35 that colocalizes with SNX1 at the PVC (Figure 3B).

Mutants in the *VPS29* gene, which has been shown to be crucial for protein storage vacuole formation in *Arabidopsis* embryos [34], exert conditional growth arrest phenotypes on sucrose-depleted medium (Suppl. Figure 3). Similarly, *snx1* seedlings exhibited a pronounced growth arrest on sucrose-depleted medium (Figure 3D). In the most severe cases, *snx1* mutant seedlings arrested growth after cotyledon formation (Figure 3D). The growth arrest of sucrose-depleted *snx1* seedlings was conditional and could be fully rescued by sucrose application (Figure 3F). Remarkably, similar growth phenotypes have been described for mutants in *GRAVITROPISM DEFECTIVE 2 (GRV2)*, an *Arabidopsis* homolog of *RECEPTOR MEDIATED ENDOCYTOSIS 8* from *Caenorabditis elegans* [35]. *grv2* mutants are characterized by deficiencies in shoot gravitropism and exhibit a conditional growth arrest phenotype, when grown in the absence of sucrose [35]. These *grv2* phenotypes have been attributed primarily to defects in the late steps of the endocytic pathway that interfere with the proper delivery of cargo to storage as well as to lytic vacuoles [35, 36].

Hence, we assume that interference with storage vacuole formation affects the energy consuming postembryonic leave organ formation, leading to growth arrested phenotypes that can be rescued by exogenous sucrose. The analogy to the grv2 growth phenotype could indicate that the SNX1/VPS29 dependent retromer complex is involved protein storage vacuole formation and in late steps of the endocytic pathway for lytic transmembrane protein degradation.

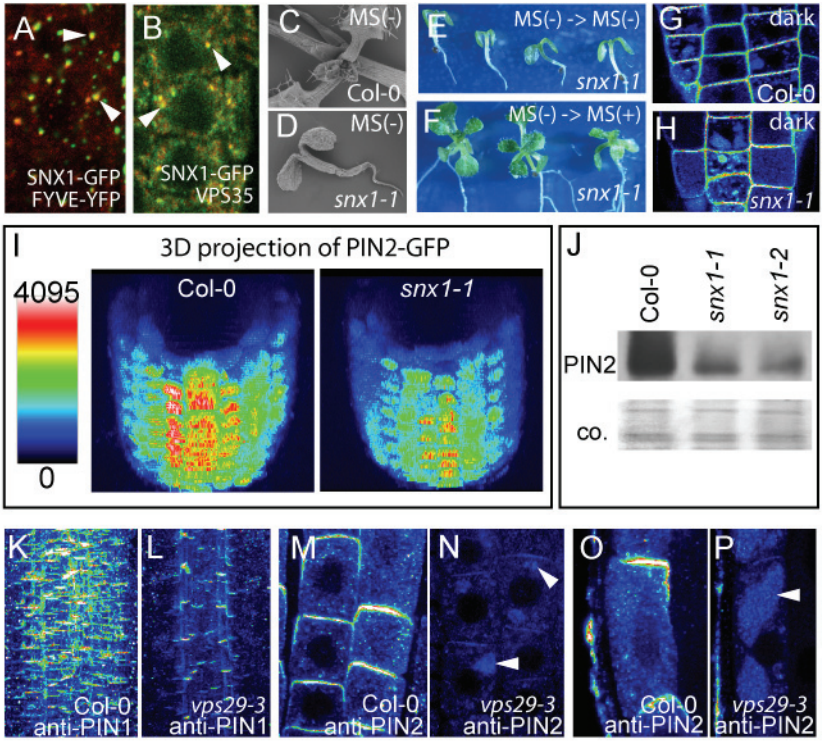


Figure 3. Putative plant retromer components regulate rate of PIN degradation.
(A-B) Merged images of colocalization (arrowheads) of SNX1-GFP (green) with the PI3P-binding domain FYVE-YFP (red) (A) and retromer component VPS35 (red) (B).
(C-F) Two-week-old wild-type seedlings with normal growth on media without sucrose (C), and *snx1* mutants arrested in growth (D). Rescue of growth defects by transfer on sucrose containing media (F).
(G-H) Substantial vacuolar targeting of the PIN2-GFP in the wild type (G) and even more pronounced in *snx1* (H) mutant after dark treatments for 2 h.
(I) Reduced PIN2-GFP levels at the plasma membrane in *snx1* mutant. Three-dimensional-animation of z-stacks (80 µm with 2-µm steps) were obtained. Roots were digitally tilted for outlook at the apical cell surface.

False color code was used for PIN2-GFP intensity visualization.
(J) Reduced total PIN2 protein levels in *snx1* mutants by quantitative western analysis.
(K-L) Reduced PIN1 protein levels in *vps29* (L) compared to wild type seedlings (K) by z-stack analysis and maximum projection of the PIN1 immunolocalization (approximately 80 µM, 2-µM steps).
(M-P) PIN2 immunolocalization in wild-type (M and O) and *vps29-3* mutants (N and P). PIN2 accumulation in *vps29* was ectopically localized in vacuolar-like structures in meristematic (I') and in elongated root cells (J'), suggesting enhanced degradation in vacuoles.
Arrows indicate BFA-dependent accumulation and arrowheads the PIN occurrence at the plasma membrane. False color code depicts relative fluorescent intensity (I and K-P)

The putative retromer components SNX1 and VPS29 mediate vacuolar targeting and steady state levels of PIN proteins

The similarity of the phenotypes observed in either *vps29* or *snx1* might indicate overlapping, interdependent functions of the corresponding gene products in the control of vacuolar targeting. Therefore, we analyzed abundance and intracellular distribution of the PIN proteins in these mutants. Mutants in *snx1* interfere with steady-state PIN2 levels, reflected in reduced PIN2-GFP abundance at the epidermal plasma membrane and in diminished PIN2 protein levels in *snx1-1* and *snx1-2* membrane protein preparations (Figure 3I and 3J). Changes in PIN protein abundance in *snx1* mutants were independent of transcriptional regulation and hint at a posttranslational regulation (data not shown). Similar observations were made, when analysing endogenous PIN1 and PIN2 in a *vps29* mutant, demonstrating reduced amounts of both proteins at the plasma membrane and elevated amounts of PIN2 signals in vacuole like structures, beeing suggestive for enhanced turnover of the protein (Figure 3K-P). Moreover, our data indicates that retromer dependent sorting events at the PVC affects both apical and basal PIN2 or PIN1 targeting.

PIN proteins have been shown to constantly cycle between plasma membrane and an endosomal pool whose identity is not fully understood yet [2, 3, 23]. It has been suggested that the putative retromer complex is required for polar recycling of PIN proteins by regulating PIN exocytosis to the plasma membrane downstream of GNOM [33]. Thus, disturbance of the exocytotic branch of PIN cycling could also lead to observed changes in PIN abundance at the plasma membrane in *snx1* or *vps29* by default

targeting to the vacuole. However, even prolonged pharmacological interference with the SNX1 labelled PVCs did not inhibit PIN targeting to the plasma membrane (data not shown; Suppl. Figure 4D; 4H). In contrast, BFA treatment leads to reversible GNOM dependent accumulation of basal PIN cargos in agglomerating endosomes and has been extensively used to study recycling of PIN proteins [2, 23]. To investigate especially polar recycling from endosomal compartments to the basal plasma membrane in *snx1*, we used BFA washout experiments in the presence and absence of the protein biosynthesis inhibitor cycloheximide. These experiments demonstrated that the PIN polar localization, BFA response, and recycling/exocytosis from endosomes to the plasma membrane after BFA removal remains unaffected in *snx1* mutants (Suppl. Figure 4A-H; data not shown). This finding contradicts previous assumptions that the retromer complex at the PVC is involved in polar PIN exocytosis downstream of GNOM and illustrates that PIN2 and PIN1 recycling does not directly require the function of the retromer complex. Thus, while SNX1 activity appears crucial for PIN2 homeostasis, it appears not to be directly involved in polar localization or in a exocytic recycling step of PIN proteins to the plasma membrane, illustrating again the interdependent but distinct regulation of PIN recycling and vacuolar degradation.

Remarkably, deficiencies in putative plant retromer components result in defects in plasma membrane protein localization and turnover that differ from those observed upon wortmannin treatments. Specifically, while the pharmacological inhibition of the SNX1-labelled PVC by wortmannin reduced PIN2 accumulation in the vacuole, loss of SNX1 function did not but preferentially enhanced vacuolar translocation(Figure 3G-H). Moreover, unlike wortmannin treatments that lead to increased PIN2 protein levels, PIN2-GFP abundance at the epidermal plasma membrane and total PIN2 protein levels were reduced in *snx1* mutants (compare Figure 2N and Figure 3J). Moreover, in contrast to wortmannin treatments (Suppl. Figure 2G), endocytic FM4-64 uptake was not significantly altered in *snx1* mutants (data not shown). Thus, while the effects of wortmannin on the PIN localization and turnover might arise from a more general interference with the sorting of plasma membrane proteins at the PVC, such as multi vesicular body formation, the mutant analysis was consistent with a more specific function of SNX1 and VPS29 in the control of vacuolar targeting. In alignment, PIN2 targeting remained sensitive to wortmannin treatment in snx1 mutants (data not shown). The *snx1* mutant phenotypes, as well as SNX1 subcellular localization, suggest its involvement in vacuolar sorting

events at the PVC. In such a scenario, SNX1 might function in the plant retromer complex, which is required to identify a subpopulation of pre-vacuolar compartments for lytic vacuole targeting. Thus, SNX1/VPS29 seem to have a gating function for endocytic translocation of PIN2 to the lytic vacuole for degradation.

Differential PIN2 degradation in lytic vacuoles in response to external stimuli

Posttranslational mechanisms that determine protein abundance of the PIN auxin efflux carriers and their contribution to the plant development are still ill defined. External signals such as light or gravitropic stimulation have been reported to trigger intracellular redistribution to the vacuole or increased degradation of PIN2, respectively [14, 16]. Quantitative western blot analysis revealed that the total PIN2 protein levels are downregulated after both prolonged dark treatment (Suppl. Figure 5) and gravity stimulation (Suppl. Figure 6). In particular, the PIN2 is downreguled in response to gravity stimulus only in a transient manner and recovers after prolonged stimulation, suggesting a defined and complex regulation of the PIN2 turnover in response to external stimuli. To obtain further insights into the biological role of proteolytic PIN2 turnover, we employed vacuolar targeting visualization methods to examine the differential degradation of plasma membrane proteins in response to a gravity signal.

To investigate whether the gravity stimulus induces PIN2 degradation via this pathway, we utilized dark treatments to monitor PIN2 targeting to the lytic vacuole. Notably, in PIN2-GFP seedlings, higher GFP accumulation was detectable in vacuoles of epidermis cells at the upper side of gravity-stimulated roots (Figure 4A-D), suggesting rather a spatial, differential regulation of the vacuolar PIN2 trafficking during gravitropism. This finding appears to question our previous findings that PIN2 degradation depends on proteasom activity [14]. Notably, pharmacological inhibition of proteasom function interfered with vacuolar translocation of PIN2 (data not shown), indicating a indirect mode of action. This finding substantiates previous reports on asymmetric PIN2 stability [14] and indicates involvement of differential vesicle trafficking of PIN2 to the lytic vacuoles during the gravitropic response. In this scenario, the asymmetric vesicle transport for vacuolar degradation of PIN2 with an increased activity at the upper side of gravistimulated roots limits the PIN2 activity and, hence, auxin flux into the elongation zone, eventually inducing differential cell elongation that results in root bending towards the gravity

vector.

SNX1 is required for the temporal PIN2 degradation during the gravitropic response

Interestingly, PIN2 preferentially resides in SNX1-labelled PVC after gravity stimulations [4] and, moreover, *snx1* mutants are defective in gravitropic response [4]. As differential degradation of PIN2 in lytic vacuoles regulates gravitropic responses (Figure 4A) [14], we analyzed potential SNX1 involvment in this process. PIN2 showed gravity-induced differential downregulation of upper epidermal cell files in wild type and *snx1* mutants (Figure 4E). To address potential quantitative differences we monitored PIN2 protein levels after gravity stimulation. Quantitative western analysis revealed that the tight regulation of the PIN2 degradation and subsequent replenishment were altered in *snx1* mutants (Figure 4F), suggesting that the SNX1 function is required for temporally defined vacuolar targeting of PIN2 after gravity stimulation and might account for the gravitropic defects observed in *snx1* mutants [4]. In this scenario, the SNX1-dependent protein retrieval from the PVC prevents the PIN2 degradation after prolonged gravitropic responses.

Our finding substantiates previous findings on gravity-induced targeting of PIN2 [4, 14] that depends on the SNX1 function. Also, it further illustrates that PIN2 gets translocated to the vacuole for degradation via the SNX1-dependent pathway during plant development. Moreover, the SNX1-dependent feedback mechanism for the PIN2 retrieval and subsequent recovery for recycling after prolonged gravitropic stimulus/responses appears to be functionally important for the plant development. In this scenario, the retromer complex prevents transition of PINs through the PVC and shuffles the proteins to the recycling endosomes, thus, revealing its interdependency but also enabling independent regulation of polar targeting to the plasma membrane and posting to the vacuole for degradation.

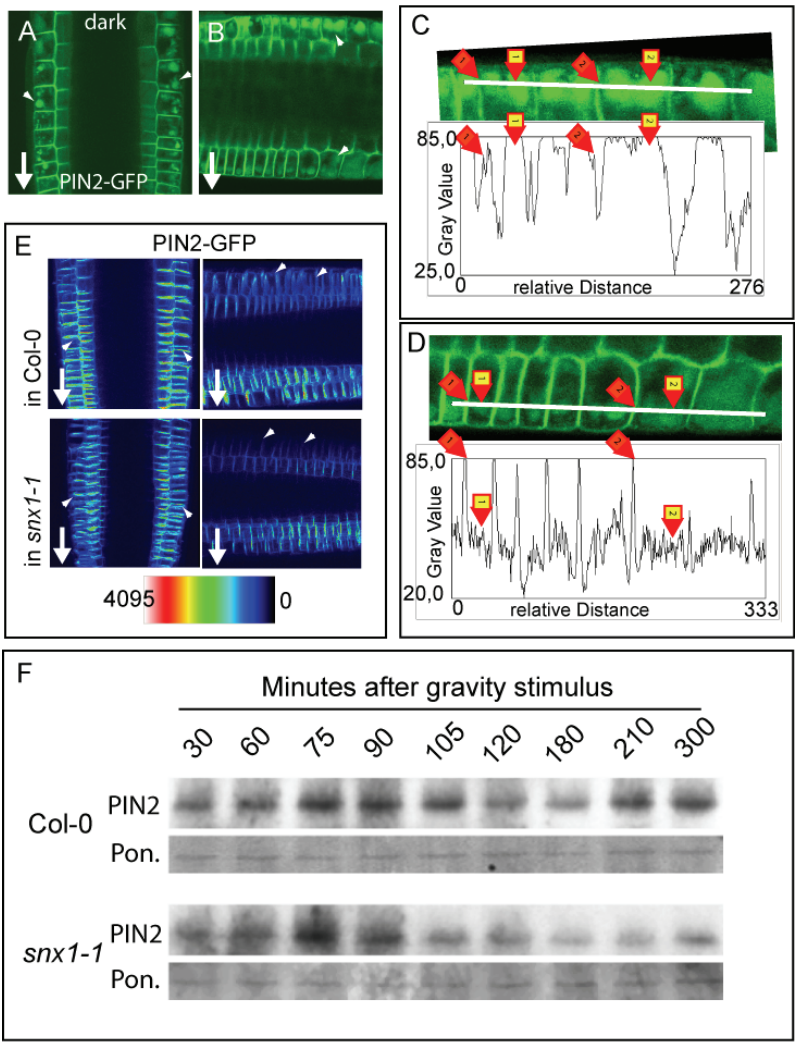


Figure 4. Differential PIN targeting to the lytic vacuole during plant development.
(A-B) Increased PIN2 targeting to the vacuole in epidermal cells at the upper root side in contrast to the symmetric vacuolar signal in vertically

grown roots (A) after dark treatment of gravistimulated [for 3 h] PIN2-GFP roots (B).
(C-D) Stronger GFP intensity in vacuole of upper epidermis cells (see yellow boxes 1 and 2). Enhanced localization in vacuoles correlated with reduced signals at the plasma membrane (red boxes 1 and 2) in upper (C) and lower (D) epidermal cell files after gravity stimulation in the dark.
(E) Differential downregulation of PIN2-GFP in upper epidermal cells after gravitropic stimulus for 3h in *snx1* mutant seedlings.
(F) Enhanced gravity-induced degradation of PIN2 in *snx1* mutants revealed by quantitative time course of total PIN2 protein abundance after gravity stimulation.
Arrowheads mark differential PIN degradation in lytic vacuoles.

Conclusions

Our data provide novel insights into the turnover mechanism of plant plasma membrane proteins, including the PIN auxin efflux carriers. The PIN proteins regulate important decisions during plant development by limiting rate and direction of the polar auxin transport [1, 5]. The PIN-dependent auxin transport can be controlled at the level of PIN transcription [7, 8], polar targeting [37-40], endocytosis [12] or protein stability [14].

We show that degradation of PIN proteins is mediated by their targeting to the vacuole that depends on the actin cytoskeleton and PI3K activity. The PIN2 vacuolar trafficking and its polar recycling to the plasma membrane are interdependent, yet molecularly distinct, thus, enabling the independent modulation of the PIN2 protein abundance and polar localization. The ARF GEF GNOM regulates the PIN recycling rate to the basal plasma membrane [2, 3], while (an) additional partially BFA-sensitive ARF GEF(s) appear(s) to regulate the vacuolar degradation of PIN2 most probably on endosomes-to-PVC trafficking level. On the other hand, activities of the putative retromer components SNX1 and VPS29 are required for defined PIN2 translocation from the PVC to the vacuole. It appears that the retromer complex promotes PIN2 retrieval from the PVC most likely to the TGN, but is not directly involved in polar PIN targeting to the plasma membrane. The PIN vacuolar trafficking and, henceforth, PIN levels can be controlled by external signals, such as light and gravity, providing a mechanism for translating environmental signals into modulation of intercellular auxin fluxes and, consequently, auxin-

dependent development.

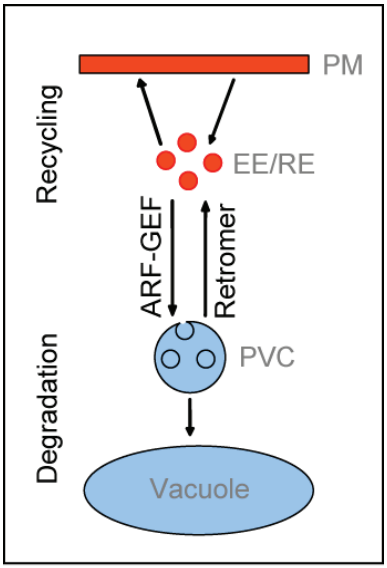
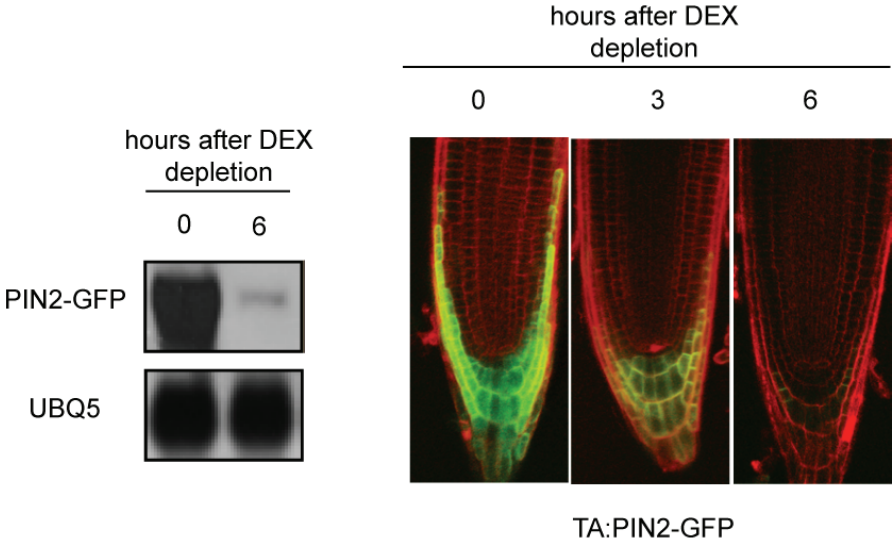
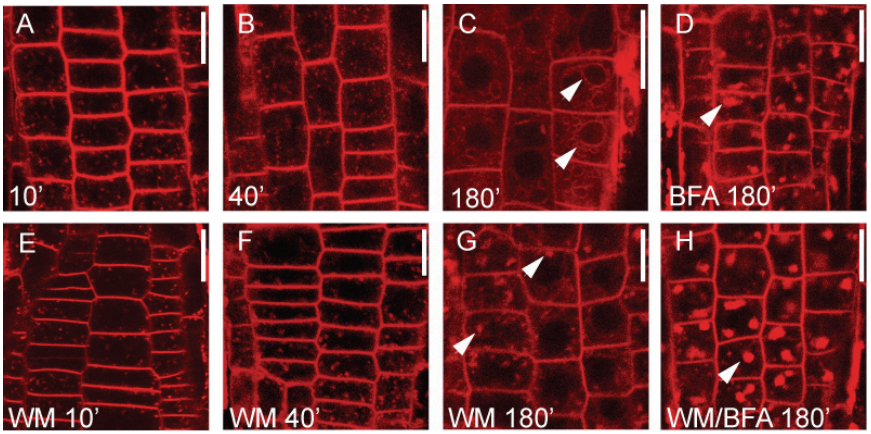


Figure 5. Model for intracellular sorting of PIN proteins.
Vesicle transport regulators such as ARF-GEF(s) and the retromer complex regulate PIN stability in a counter directional manner. Partially BFA sensitive ARF-GEF(s) appear(s) to promote PIN degradation in lytic vacuoles. In contrast, activity of the retromer complex regulates PIN retrieval from the pre vacuolar compartment (PVC), thus, keeping the PIN proteins in a subpopulation of early- or recycling endosomes (EE/RE) for subsequent targeting to the plasma membrane (PM).



Supplemental Figure 1. PIN2 protein turnover after DEX induction. DEX-induced PIN2-GFP showed rapid degradation within hours. PIN2 degradation was confirmed by western and confocal microscopy (PIN2-GFP in green; propidium iodide in red was used as counter staining)

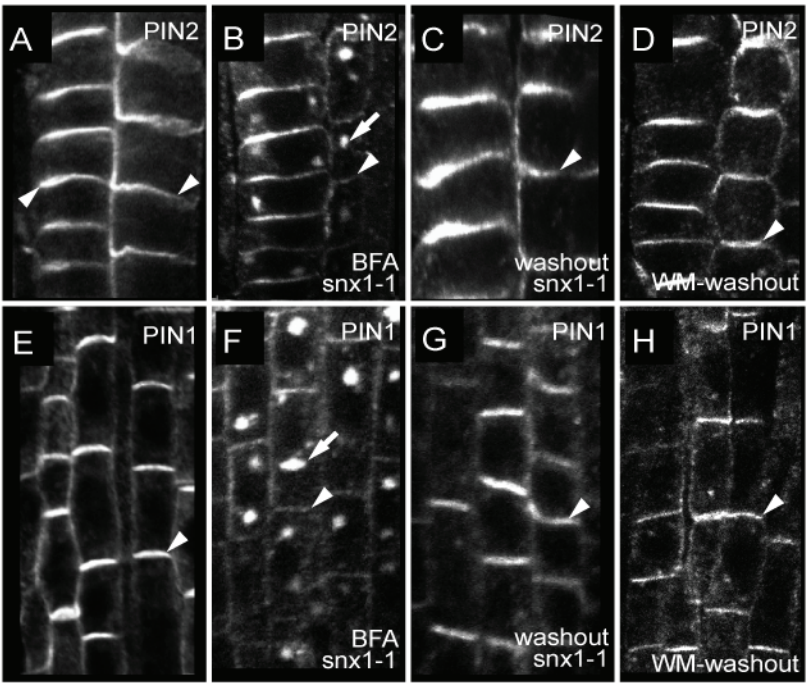


Supplemental Figure 2. Effect of wortmannin on the intracellular

membrane flow to the vacuole.
(A-C) FM4-64 uptake in root epidermal cells of untreated wild-type seedlings after 10 (A), 40 (B), and 180 (C) min.
(D) Disruption of FM4-64 targeting to the tonoplast after BFA [50 µM] treatment.
(E-G) FM4-64 uptake in root epidermal wild-type root cells treated with wortmannin [15 µM] after 10 (A), 40 (B) and 180 (C) min; initially, wortmannin did not visibly interfere with FM4-64 uptake, but displayed severely reduced FM4-64 trafficking to the tonoplast.
(H) FM4-64 accumulation in BFA compartments by concomitant treatments of wortmannin [15 µM] and BFA [50 µM], illustrating ongoing endocytosis in the presence of wortmannin.

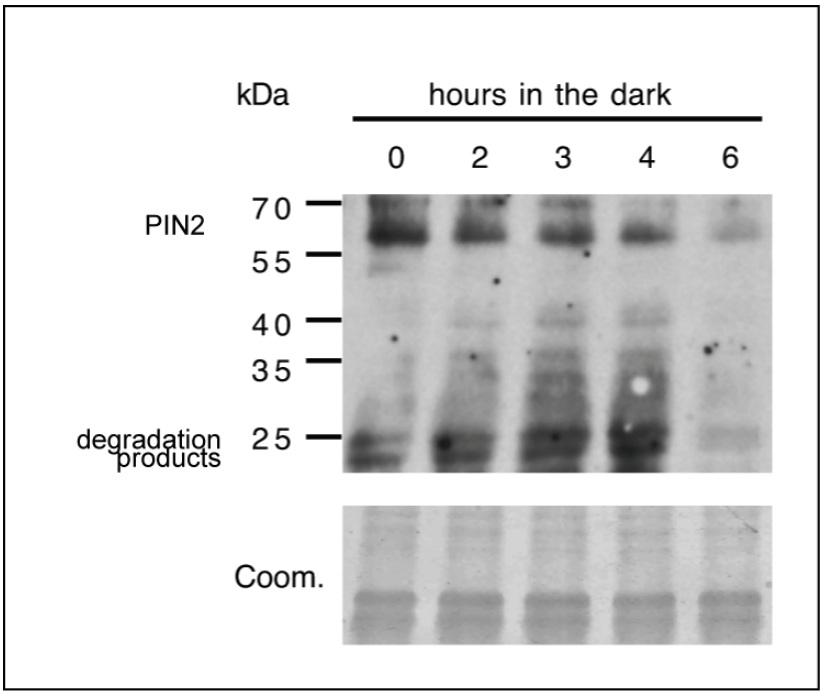
Allele	growth inhibited phenotype	
	without sucrose	with sucrose
Col-0	4%, n=83	0%, n=55
snx1-1	26%, n=75	0%, n=45
vps29-3	95%, n=36	0%, n=32

Supplemental Figure 3. Conditional growth phenotype in the absence of exogenous sucrose.
Percentage of wild-type, *snx1*, and *vps29* mutants that displayed severely inhibited growth in the absence of exogenously applied sucrose.

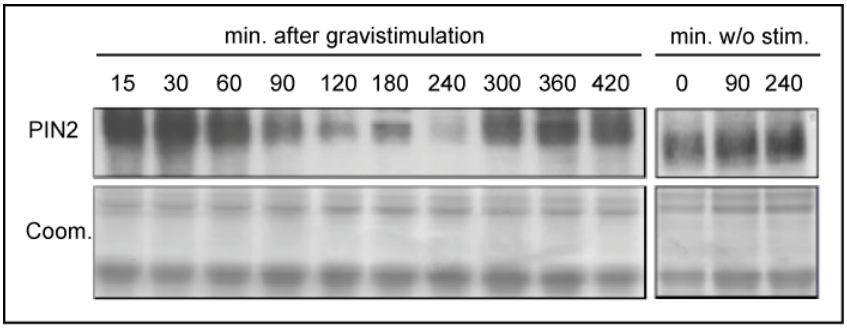


Supplemental Figure 4. SNX1 is not directly involved in polar PIN targeting or recycling.
(A-D) PIN2 immunolocalization in *snx1-1* mutants. PIN2 localization at the apical plasma membrane in epidermal and basal in cortex cells (A), suggesting unaffected polar PIN targeting in *snx1* mutants. BFA [50 µM, 1 h] induced rapid internalisation of basally localised PIN2 in cortex cells (B), indicating constitutive endocytosis of PIN2 in *snx1* mutants. PIN2 recycled back to the plama membrane following BFA removal [1 h washout] (C), revealing no polar targeting defects in the *snx1* mutant background. Pharmacological impairment of SNX1-labelled PVCs by wortmannin [15 µM] did not prevent recycling of PIN2 following BFA removal (D). Differences to wild-type samples were not observed (data not shown).
(E-H) PIN1 immunolocalization in *snx1-1* mutants. Basal PIN1 in the stele was unchanged (E). BFA [50 µM, 1 h] induced rapid internalisation of PIN1 (F) that recycled back following BFA removal [1 h washout] (G). BFA removal in presence of wortmannin [15 µM; 1 h] did not prevent recycling

of PIN1 to the plasma membrane (H). Differences to wild-type samples were not observed (data not shown).
Arrows mark BFA-dependent accumulation and arrowheads the PIN occurrence at the plasma membrane. Abbreviations: C, cortex; E, epidermis cells.



Supplemental Figure 5. PIN2 protein degradation in dark-grown seedlings.
Quantitative Western analysis revealed dark-dependent degradation of total PIN2 after 2-4 h.



Supplemental Figure 6. Gravity-induced temporal degradation and recovery of PIN2.
(A) Time dependent down regulation of total PIN2 proteins after gravity stimulation (left panel) and no differences in protein abundance in unstimulated samples (right panel) revealed by quantitative Western analysis.

Materials and Methods

Materials

PIN1:PIN1-GFP [38]; PIN2:PIN2-GFP(1) [20]; PIN2:PIN2-GFP(2); TA:PIN2-GFP [14]; BRI1:BRI1-GFP(1) [21, 41]; BRI1:BRI1-GFP(2) [22]; 35S:PIP2-GFP [42]; *snx1-1*, *snx1-2* and SNX1-GFP, SNX1-RFP [4]; *vps29-3* (obtained from SALK) [33]; and YFP-2xFYVE [43] have been described previously. Data presented in the figures were all obtained with PIN2:PIN2-GFP(1) and BRI1:BRI1-GFP(1). Control experiments with PIN2:PIN2-GFP2 and BRI1:BRI1-GFP2 led to similar results.

Growth conditions

Plants were grown on soil or Murashige and Shoog (MS) plates (with or without sucrose) as described [10] under a 16 h light/8 h dark photoperiod at 21/18°C. Dark experiments were carried out in light period.

Drug applications and experimental conditions

Exogenous drugs were applied by incubation of 5-day-old seedlings in liquid half-strength MS medium supplemented with BFA (50 mM stock in dimethylsulfoxide [DMSO]) (10/25/50 µM), cycloheximide (50 mM stock in DMSO) (50 µM), concanamycin A (1 mM stock in DMSO) (1 µM),

latrunculin B (20 mM stock in DMSO) (20 μM), or wortmannin (30 mM stock in DMSO) (15 μM). Control treatments contained an equivalent amount of solvent (dimethylsulfoxide). Double drug treatments were carried out with 30 min of pretreatment followed by concomitant drug treatment for the indicated time.

Dark treatments were done in liquid or solid MS medium. For gravitropic analysis, a dark treatment for 2 h was performed followed by gravistimulus for 3 h.

FM4-64 uptake experiments were performed as described [12].

For all comparisons, independent experiments were done at least in triplicate with the same significant results and representative images are presented. Statistics were evaluated with Excel (Microsoft office 2003).

Expression and immunolocalization analyses

Wholemount immunofluorescence was prepared as described [38]. Antibodies were diluted as follows: 1:500 anti-GFP (Molecular probes), 1:2000 for anti-PIN2 [14]; 1:2000 for anti-PIN1 [12]; 1:500 for anti-VPS35 (generously provided by D. Robinson) and 1:500 and 1:600 for FITC- and CY3-conjugated anti-rabbit/mouse secondary antibodies (Dianova), respectively. GFP was visualized in 5% glycerol without fixation for live cell imaging. For confocal laser scanning microscopy, a Leica TCS SP2 AOBS, Zeiss CLSM, and Olympus FV10 ASW were used. The intensity of the PIN2-GFP signal was measured with ImageJ 1.37v. Images were processed in Adobe Photoshop CS2 (Adobe Inc.).

The PIN2 protein levels in membrane protein fractions were analysed as described previously [14]. Affinity-purified anti-PIN2 was decorated with HRP-conjugated goat anti-rabbit IgG (Pierce, USA; 1:100.000) and detected by enhanced chemiluminescence (SuperSignal West Pico, Pierce).

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Investigator Program (to J.F.) and grants from the FWF (P19585) and the WWTF (LS0535) (to C.L.). We disclose any financial conflict of interest that might be construed to influence the results or interpretation of the manuscript.

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Chapter 6

Concluding Remarks:

Author's Contribution to the Manuscripts:

Kleine-Vehn J and Friml J (2008) Polar Targeting and Endocytic Recycling in Auxin-Dependent Plant Development. Annu. Rev. Cell Dev. Biol. 24:447-73.

JKV assembled the figures; JKV and JF discussed the outline and wrote the manuscript.

Kleine-Vehn J, Dhonukshe P, Swarup R, Bennett M, Friml J. 2006. A novel pathway for subcellular trafficking of AUX1 auxin influx carrier. *Plant Cell* 18:3171—81.

JKV carried out most of the experiments; PD performed experiments presented in Figure 1H, I, 3A, C, 5J,K; RS contributed Figure 4A,B; MB helped in finalizing the manuscript; JKV assembled the figures; and JKV and JF designed the experiments, discussed the results and wrote the manuscript.

Kleine-Vehn J, Dhonukshe P, Sauer M, Brewer PB, Wiśniewska J, Paciorek T, Benková E, Friml J. 2008a. ARF GEF-dependent transcytosis and polar delivery of PIN auxin carriers in *Arabidopsis*. *Curr. Biol.* 18:526—31.

JKV initiated the project; designed most of the experiments and carried out most of the experiments; PD performed experiments presented in Figure 3B-C; MS performed embryo analysis (Figure 4A-E); PBB and JW contributed to Figure 1; TP analysed Figure S1C-E; EB performed analysis on lateral root primordia (Figure 4F-K); JKV assembled the figures; PBB helped in finalizing the manuscript; and JKV and JF discussed the results and wrote the manuscript.

Kleine-Vehn J, Langowski Ł, Wiśniewska J, Dhonukshe P, Brewer P, and Friml J. (2008) Cellular and molecular requirements for polar PIN targeting and transcytosis in plants. *Submitted.*

JKV initiated the project, designed most of the experiments and carried out most of the experiments; ŁL performed experiments for Figure 1; JW contributed to Figure 2; PD performed experiments for Figure 4E; PBB analysed data for Figure 3G-I; JKV assembled

the figures; JKV and JF discussed the results and wrote the manuscript.

Kleine-Vehn J, Leitner J, Zwiewka M, Sauer M, Abas L, Luschnig C and Friml J. (2008) Differential degradation of PIN2 auxin efflux carrier by SNX1-dependent vacuolar targeting. *Submitted.*

JKV initiated the project, designed most of the experiments and carried out most of the experiments, JL performed the western analysis shown in Figure 2N; 3J; 4F; S1; S5, experimental contribution of MZ is not represented in the figures, MS ideas were important for the initiation of the project, LA did the gravity western for Figure S6, JKV assembled the figures, MS and CL helped in finalizing the manuscript, and JKV and JF discussed the results and wrote the manuscript.

Frequently used abbreviations:

ARF	Adenosyl ribosylation factor
AUX1	AUXIN-RESISTANT1
BFA	Brefeldin A
BRI1	BRASSINOSTEROID INSENSITIVE 1
CHX	Cycloheximide
Col-0	Columbia-0
ConA	Concanamycin A
DMSO	Dimethylsulfoxide
ER	Endoplasmatic reticulum
FM4-64	N-(3-triethylammoniumpropyl)-4-(p-diethylaminophenylhexatrienyl) pyridinium dibromide
GEF	Guanine nucleotide exchange factor
GFP	GREEN FLUORESCENT PROTEIN
GUS	Glucuronidase
IAA	Indolacetic acid
LatB	Latrunculin B
LAX	LIKE-AUX1
MS	Murashige and Shoog
MVB	Multi vesicular body
NAA	Naphthalenacetic acid
1-NOA	1-naphthoxy acetic acid
NPA	1-N-naphtylphtalamic acid
PBA	Phthalamic acid
PID	PINOID
PI3K	Phosphatidylinositol-3-kinase

PIN	PIN-FORMED
PIS1	POLAR AUXIN TRANSPORT INHIBITOR-INSENSITIVE1
PM	Plasma membrane
PVC	Pre vacuolar compartment
SMT1	STEROL METHYLTRANSFERASE1
SNX	SORTING NEXIN
TGN	Trans Golgi network
TIBA	2,3,5-triiodobenzoic acid
VPS	VACUOLAR SORTING PROTEIN
WM	Wortmannin

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PUBLICATIONS

Kleine-Vehn J and Friml J (2008) Polar Targeting and Endocytic Recycling in Auxin-Dependent Plant Development. Annu. Rev. Cell Dev. Biol. 24:447-73.

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My last task…
It is done. My official education finishes here. However, I hope learning will never finish, but now is the time to look back. I did well, but only because nice people put me on track.
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I am very happy that I could meet, discuss, and sometimes just fall silent with so many nice personalities.

Probably about seven years ago I run into a very gifted postdoc during one of my practical student courses. He showed me right away that science is hard work, but also exciting. Step by step, he persuaded me to do plant science. He was really good in doing so, I even realized too late that I actually started a PhD with him. Jiri, thanks a bunch! Without you my scientific understanding would not be the same. I learned so many things even by just seeing you performing. I am truly grateful for everything you did for me; for your constant support, the fruitful (and sometimes “wineful”) discussions, the casual chats and the freedom.
I will never forget this nice time and for example my progress regarding my writing skills…
Jiri: ”Jürgen, it is dreadful! How much time did you spend, an hour?”
> I probably worked on the draft for about two weeks…
Jiri: “I was surprised, it is not so bad!”
> I still believe he did not read this particular manuscript ;)
Dear promoter, it was fun and I am honoured to be your first official PhD!

It sounds a bit artificial to acknowledge somebody for creating a special atmosphere, but nevertheless I do and am grateful to Gerd Jürgens for the fantastic time in his department especially in the beginning. Quite often we need time to understand. Attending seminars with extraordinary scientist such as Eva Benkova, Niko Geldner, Dolf Weijers, Jiri Friml and Gerd Jürgens appeared almost normal to me at that time. I was just a student who attended to the department seminars on a voluntary basis and every week I could experience people that do not simply perform, but are fascinated by science. It truly affected me and my relation to do science and it is difficult to state how valuable this time was for my early education.

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