# Regulation of PIN-FORMED Protein Degradation

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Auxin action largely depends on the establishment of auxin concentration gradient within plant organs, where PIN-formed (PIN) auxin transporter-mediated directional auxin movement plays an important role. It has revealed the need of polar plasma membrane (PM) localization of PIN proteins as well as regulation of PIN polarity in response to developmental cues and environmental stimuli, amongst which a typical example is regulation of PIN phosphorylation by AGCVIII protein kinases and type A regulatory subunits of PP2A phosphatases.

PIN-FORMED degradation vacuolar sorting ubiquitin modification

## 1. Introduction

PIN-formed (PIN) proteins are auxin efflux carriers that are extremely important for auxin-triggered organogenesis in plants. Early in 1998, it was uncovered that polar plasma-membrane localization behaviors of PIN proteins mediate polar auxin transport during inflorescence development and root gravitropism of *Arabidopsis thaliana* [1][2]. In total, eight members the PIN family are found in Arabidopsis. All of them typically have a central hydrophilic loop between two hydrophobic regions of five transmembrane domains each. Based on length of the central hydrophilic loop, PINs are generally divided into two groups: long-type PINs (PIN1–PIN4 and PIN7) that localize at the PM and regulate intercellular auxin transport as auxin efflux facilitators [3]; short-type PINs (PIN5 and PIN8) that localize at the endoplasmic reticulum (ER) and contribute to homeostasis [4][5][6]. PIN6, processing a middle length of the hydrophilic loop between long- and short-type PINs, shows dual localizations at both the PM and ER depending on its phosphorylation states and expression levels [7][8]. Polarity of PM-located PINs remodels auxin efflux behavior then controls directional cell-to-cell auxin transport in tissues to establish auxin gradient for the following activation of auxin signaling.

PINs, like other PM-localized transmembrane proteins, are distributed along with their resident vesicle bodies through the intracellular trafficking system. Establishment of PIN polarity involves delivery of new synthesized proteins to the PM by exocytosis and removal from the PM by endocytosis [9]. Once processes are disturbed among sorting, trafficking, and turnover (synthesis and degradation) of membrane proteins, the PIN abundance and polarity are affected. The intracellular trafficking of PINs most depends on the action of guanine nucleotide exchange factors for ADP ribosylation factors (ARF GEFs), including GNOM and GNOM-like1 (GNL1), and BFA-visualized endocytic trafficking defective (BENs) [10][11][12][13]. PIN phosphorylation regulated by AGCVIII protein kinases, such as PINOID, and by type A regulatory subunits of PP2A phosphatases, for example PP2AA1/RCN1, is a well-studied mechanism for repolarization of PIN localization [14][15].

## 2. PIN Degradation

Turnover of proteins is crucial to maintain their normal function and to allow their levels to change quickly in response to stimuli in organisms. Membrane proteins, including PINs, are normally targeted to the vacuole for degradation [9]. Dark treatment makes PINs more stable in the vacuole and thus the vacuole-like accumulation of GFP-tagged PINs is visualized [16]. The vacuole-targeted PIN proteins can also be observed by treatment with concanamycin A (ConcA) [16], a vacuolar $H^+$-ATPase inhibitor that neutralizes vacuolar pH and thus blocks trafficking to the vacuole [17]. Wortmannin, a PI3K and PI4K inhibitor [18], displays a similar inhibitory role in vacuolar targeting process of certain PINs in some special scenarios by forming different aggregations called WM compartments, also suggesting dependence of PIN vacuolar trafficking on PI3K activity [16]. In contrast, trafficking and endocytosis inhibitor 1/TENin1 (TE1) reversibly inhibits retrograde trafficking of membrane cargos (e.g., PIN2-GFP) from the multivesicular body (MVB) to the trans-Golgi network (TGN) and thus promotes vacuolar targeting, whilst displays an inhibitory effect on endocytic recycling [19].

To date, PIN2 relocalization in gravitropic response is the clearly documented scenario involving PIN regulation by protein degradation. PIN2 localizes at the PM of root cortical and epidermal cell files to establish a lateral auxin gradient in the root tip [2][20]. During gravistimulation, PIN2 distribution displays differential between the upper and the lower sides of roots to establish asymmetric distribution of auxin [21], which process is largely dependent on post-transcriptional mechanism [22]. The reduction of PIN2 signal at the upper side is due to the enhanced vacuolar targeting of PIN2, as visualized by dark treatment [16]. The interference with asymmetric distribution of PIN2 by treatment of MG132, a 26S proteasome inhibitor, indicates that gravi-induced proteolysis of PIN2 depends on proteasome-dependent manner [21].

## 3. Ubiquitin Modification

Ubiquitin modification acts as a sorting signal for integral membrane proteins [23]. Numerous studies have indicated that ubiquitination of PIN-formed proteins triggers their protein degradation in the vacuole. In Arabidopsis roots, vacuolar targeting and proteolytic turnover of PIN2 are associated with PIN2 ubiquitination status [21][24], which undergoes a continuous enhancement in initially several hours after gravistimulation [25]. Although PIN2 stability and ubiquitination level depend on 26S proteasome [21], through which Lysine-48 (K-48) linked polyubiquitin chains of soluble proteins act as the canonical signal for degradation [23], K-63 ubiquitination that is required for the vacuole targeting of membrane proteins is detected as the major form of PIN2 polyubiquitination [24]. As membrane proteins cannot directly access to the proteasome, the involvement of proteasome activity may facilitate the trafficking process of PINs to the vacuole [21]. In mammalian and yeast systems, the ubiquitinated membrane proteins, in addition to direct traffic of misfolded proteins from the Golgi to endosome or subjection to ER-associated degradation (ERAD), are generally endocytosed and targeted to the lysosome/vacuole for degradation [23].

In contrast to ubiquitination, deubiquitylation has also been implicated in PIN degradation. Associated molecule with the SH3 domain of STAM3 (AMSH3), a major Arabidopsis deubiquitinating enzyme (DUB) that hydrolyzes

K48- and K63-linked ubiquitin chains but is independent of the 26S proteasome, regulates ubiquitin-mediated endocytic degradation [26][27]. Mutation in AMSH3 causes defect in vacuole formation and accumulation ubiquitinated membrane proteins, and thus results in disability of PIN2 vacuolar degradation [26]. Analogous phenotypes can be found in the mutant of *amsh1* and the plant overexpressing dominantly negative form of VPS2.1, the ESCRT-III (discussed below) subunit that interacts with AMSH1 or AMSH3 [27][28].

## 4. Ubiquitin E3 Ligases

Currently, several ubiquitin E3 ligases, which recognize and transfer ubiquitin to the substrate and thus determine substrate specificity [29], have been revealed to control PIN protein stability. Ring domain ligase1 (RGLG1) and RGLG2 act redundantly in K-63 polyubiquitination and auxin-regulated development events [30]. Reduced accumulation of PIN1-GFP, as well as PIN2-GFP, in *rglg1rglg2* roots indicates that PIN1 and PIN2 are unlikely to be direct targets of RGLGs for ubiquitination-mediated degradation, although physical interaction between RGLG2 and PIN1 was detected in the yeast-two-hybrid assay [30]. onstitutive photomorphogenic1 (COP1), a RING E3 ubiquitin ligase playing a core role in photomorphogenesis and skotomorphogenesis, regulates PIN localization in different ways. In shoots, COP1 delivers light signal to roots and influences root growth, through transcriptional reduction of PIN1 in hypocotyls, and thus, modulation of PIN1-mediated basipetal auxin transport [31]. In roots, COP1, after perception of auxin transported from shoots, participates in dark induced reduction of PM-located PIN1-GFP and PIN2-GFP, as well as vacuolar accumulation of PIN2-GFP in different posttranscriptional mechanisms, because inhibition of proteasome activity by lactacystin blocked PIN2 vacuolar targeting, whereas not the intracellular distribution of PIN1-GFP [31]. Moreover, defect of PIN2 redistribution and root reorientation during gravistimulation suggest participation of COP1 in PIN2 stability in gravitropism [31].

## 5. PI3K Complexes

PI3K activity plays pivotal roles in various cellular processes, such as endocytic trafficking and autophagy, by production of phosphatidylinositol 3-phosphate (PI3P), which provides a binding site for proteins with certain lipid binding domains, including FYVE and PX domains [32]. Two main PI3K complexes are implicated in regulation of PIN proteins. The PI3K complex I, composed of vacuolar protein sorting34 (VPS34, a membrane of Class III phosphoinositide 3-kinase family), VPS15, VPS30/ATG6/Beclin1, ATG14 and ATG38, is essential for autophagosome biogenesis [33][34], while the other PI3K complex II, consisting of VPS34, VPS15, VPS30/ATG6/Beclin1, and VPS38, is generally required for ESCRT-mediated MVB formation, autophagosome-lysosome fusion, and the function of the retromer complex, which controls endosome to Golgi retrograde trafficking [32]. Due to diverse functions of PI3K complexes and additional effect by PI3K inhibitors on phagophore formation [35], PIN degradation by endosomal sorting and autophagy is ambiguous based on current knowledge.

VPS38, as a component of PI3K complex II, has a profound role in modulating late endosome/MVB morphology [36][37]. Mutation of VPS38 resulted in abnormal cytoplasmic distributions of GFP-PIN1, -PIN2, and -PIN3 [36][37][38]. The association of VPS38 with VPS30 and VPS34, another two components of PI3K complex II, and the

colocalization of VPS38 and retromer VPS29 [37][38] indicate that the regulatory role of VPS38 on PIN localization is more dependent on PVC sorting, although *vps38* mutants showed defects in autophagy [36][37].

## 6. SNAREs

Soluble N-ethylmaleimide sensitive factor attachment protein receptors (SNAREs) mediate fusion between vesicular and target membranes. Usually, some of these transmembrane proteins reside on vesicular membranes in a single chain, while the others reside on target membranes in a three-protein complex [39][40]. The assembly of SNAREs residing on both membranes to a stable four-helix bundle leads to membrane fusion. VAM3/SYP22 is a SNARE that localizes to both the MVB and the tonoplast. Since *vam3* mutant displayed depolarization of PIN1-GFP in leaves and vacuolar aggregation of PIN1-GFP was surrounded by mRFP-VAM3 labeled tonoplast under dark treatment, it is suggested that VAM3-mediated vacuolar trafficking is required for maintenance of PIN polarity [41]. In addition, mutation in both SYP42 and SYP43 (*syp42 syp43*), two SYP4 group members of TGN-localized SNARE [40], led to defect of vacuolar accumulation of PIN2-GFP in the darkness [42], also providing an indication of involvement of SYP4 in PIN vacuolar transport. Nonetheless, the regulatory effect of SNARE SYP4 is indirect and could be attributed to the influence on trafficking between the TGN and MVBs [42].

## 7. The Retrograde Systems

The retromer is a conserved protein complex that regulates vesicular transport of transmembrane proteins from endosomes to the trans-Golgi network (TGN) and to the plasma membrane [43]. It consists of a dimer of sorting nexins (SNXs), which contains PX domains binding to phosphatidylinositol 3-phosphate (PI3P) produced by VPS34 for membrane recruitment, and a trimer of VPS26, VPS29, and VPS35, which binds endocytic receptors for cargo selection [44]. In Arabidopsis, the retromer protein VPS29, as well as the physical interactors VPS35 and VPS26, colocalize with SNX1 at the MVB and are required for MVB morphology [16][45][46]. The complex controls endocytic recycling of specific cargos, such as PIN1 and PIN2, because loss of function of VPS29 just resulted in intracellular accumulation of PIN1-GFP and PIN2-GFP, but not AUX1-YFP and GFP-PIP2a, in SNX1-labled compartments [46]. The improperly recycled PIN proteins in *vps29* mutant were then destinated for degradation [16][46]. Similar alteration of PINs is also reported in *snx1* mutants [16][47]. A posttranslational regulation is suggested in such context based on the finding that the decreased PIN2 protein levels in *snx1* mutants are independent of gene transcription [16]. Recent work reveals that SNX1 forms a complex both with phosphatidylinositol 3-phosphate 5-kinase (FAB1) and its product phosphatidylinositol 3,5-bisphosphate (PtdIns(3,5)$P_2$, which is required for the association of SNX1 with the endosome membrane) to facilitate maturation of SNX1 endosomes, and hence to impair PIN2 trafficking [48].

## 8. The ESCRT Pathway

Sorting into the intralumenal vesicles of endosomes is a common step for membrane protein degradation in vacuoles. This process is controlled by the ESCRT (endosomal sorting complex required for transport), which is

defined as a ubiquitin-dependent protein sorting pathway consisting of five distinct complexes, termed ESCRT-0, ESCRT-I, ESCRT-II and ESCRT-III, and vacuolar protein sorting4 (VPS4) [49][50]. These complexes are sequentially required to interact with ubiquitinated membrane proteins and to drive their internalization into the intraluminal vesicles of MVBs. Therefore, the components of such complexes are involved in PIN degradation in vacuoles.

TOM (target of MYB) 1-likes (TOLs) have VHS (Vps27, Hrs, and STAM) and GAT (GGAs and TOM) domains similar to components of the ESCRT-0, which is essential for recruitment of the ESCRT-1 for initiating MVB-dependent cargo sorting. They function redundantly in recognizing ubiquitinated PINs and then regulating the endocytic sorting to vacuoles for protein degradation [51]. Another ESCRT-0 subunit identified in plants is FYVE domain protein required for endosomal sorting1 (FREE1), which binds to PI3P, ubiquitin, and specifically interacts with VPS23A and VPS23B, subunits of the ESCRT-I complex, via PTAP-like tetrapeptide motifs [52]. Mutation in FREE1 led to defects in MVB formation and mislocalization of vacuole-accumulated endocytosed PIN2 to the tonoplast [52]. To some extent, the ESCRT-0 together with ubiquitin modification determines specificity of vacuolar targeting of membrane proteins, because multiple mutation of TOLs caused defect in forming WM compartments of PIN2-VENUS while not in that of FM4-64 labeled endomembrane [51]. It is noteworthy that FREE1 does not merely regulate protein sorting to the vacuole but also autophagic degradation because of association between FREE1, SH3P2 (a unique plant autophagy regulator), and the PI3K complex I subunit ATG6 [53]. ELC is an Arabidopsis homolog of VPS23 displaying the binding activity to ubiquitin. It forms the ESCRT-I complex with homologs of VPS37 and VPS28 [54]. VPS28A and VPS28B are redundant components of the ESCRT-I complex involved in Arabidopsis embryonic development. In embryos of *vps28avps28b* double mutant, PIN1-GFP displayed broadened expression profile, reduced polarity, and aberrant vacuole-like structures [55]. Aberrant localization and polarity of PIN1-GFP and PIN2-GFP are also reported in the knockout mutant of VPS36, the ESCRT-II subunit that can bind ubiquitin and interact with the other ESCRT-II components VPS22 and VPS25 [56].

## 9. Cytoskeletons

Both microtubule and actin cytoskeletons that are responsible for intracellular vesicular trafficking have been implicated in regulating PIN targeting to the vacuole. Especially for filamentous actin (F-actin), auxin transporter inhibitors, such as 2,3,5-triiodobenzoic acid (TIBA) and 2-(1-pyrenoyl) benzoic acid (PBA), also serve as actin stabilizers and repress subcellular motility in plant cells [57]. Nevertheless, the inhibition of PIN2 vacuolar targeting by latrunculin B (LatB)-induced actin depolymerization [16] implies a different effect of F-actin that the stabilization may promote vesicular trafficking to the vacuole. This finding is supported by appearance of reduced PM abundance and increased intracellular aggregation of PIN2-GFP in *act7*, the loss-of-function mutant of actin subunit ACT7 with excessive actin bundling [58]. The contradiction is possibly due to inconsistent effects of various inhibitors on consequently F-actin dynamics or different roles of the actin cytoskeleton in subcellular trafficking in different scenarios. Comparing to the F-actin, few studies report the role of microtubules in PIN vacuolar sorting. Given that a microtubule-associated protein cytoplasmic linker associated protein1 (CLASP1) positively regulates microtubule depolymerization, the interaction with retromer component SNX1 indicates involvement of microtubules in PIN2 retrograde trafficking from endosomes [59].

## 10. Plant Hormones

Plant hormones are critical regulators for PIN degradation. The most studied is auxin as shown in the experiment that treatment with either naphthylene-1-acetic acid (NAA), an artificially synthesized auxin, or 2,3,5-triiodobenzoic acid (TIBA), a polar auxin transport inhibitor, reduced PIN2 protein levels with unaffected transcription levels [21][60]. The scenario of NAA-induced PIN2 degradation is further specified to PIN2 recovery decrease at the lower side of gravistimulated roots after the initial increase of PIN2 level. Meanwhile, at the upper side of the bending roots where PIN2 levels initially decrease, auxin depletion also promotes PIN2 vacuolar targeting for degradation [22]. Both scenarios of PIN2 turnover regulation involve $SCF^{TIR1/AFB}$-based auxin signaling pathway and suggest optimal auxin levels for the stabilization of PIN2 proteins [22][60]. Nonetheless, no auxin signaling components have been revealed with differential expression between the upper and lower part of root during gravistimulation, also indicating that the initial PIN2 degradation there might not be triggered by the lack of auxin. Ubiquitin modification, at least that of PIN2, might prompt new thinking for insight into regulatory mechanism of PIN degradation.

Cytokinin, frequently having crosstalk with auxin, acts as a PIN regulator at both transcriptional and post-translational levels. Take PIN1 for example, B-type ARR cytokinin response factors bind directly to the responsive elements, including PIN1 cytokinin response element (PCRE1), in PIN1 promoter to upregulate PIN1 expression in inflorescences [61]. However, in terms of regulation of PIN degradation, cytokinin, during development of lateral root primordium, targets PIN1 to the vacuole for degradation through modulation of the endocytic trafficking. Interference of the cytokinin effect by latrunculin B-caused actin depolymerization, not by that of oryzalin-induced microtubule depolymerization, suggests the mediation of the actin cytoskeleton in the process [62].

## 11. Environmental Stimuli

Gravity is the most mentioned trigger of PIN degradation, which has been discussed regarding PIN2 redistribution in root gravitropism. The perception of the gravity vector is tightly associated with statoliths, dense starch-filled organelles which settle to the vicinity of the plasma membrane of the columella of the root cap and the endodermal cells of aerial tissue under gravistimulation [63]. Although the physical attachment of amyloplasts to the cytoskeleton and activation of calcium release from the endoplasmic reticulum by statoliths suggest the signal transduction during gravitropic response and reorient plant growth via auxin [64], the detailed link of gravity perception to the establishment of auxin gradient remains unidentified to date. In addition, light negatively regulates PIN2 vacuolar targeting in HY5-dependent pathway, but the mechanism is still large in the shadow and 26S proteasome and COP9 signalosome, which directly modulates ubiquitin E3 ligase function for26 proteasome-mediated degradation, are involved in this process [65].

## 12. Other Regulators

In addition, some other genes are reported to regulate PIN degradation. MODULATOR OF PIN2 (MOP2) and MOP3, which are identified by a genetic screen, control PIN stability in a nonredundant manner as mutation of

either reduced PIN protein levels [66], however, the underlying mechanism of these newly characterized PIN regulators is still unknown. During establishment of procambial cells in cotyledons, expression and localization are under the regulation of vasculature complexity and connectivity (VCC), a plant-specific transmembrane protein which expression is induced by auxin. As VCC mutated, depolarization and vacuolar accumulation of PIN1-GFP were enhanced [67]. SSR1 is a mitochondrial protein that regulates the retrograde trafficking possibly through the influence on the retromer. Knock-out of SSR1 resulted in decrease of protein expression of canonical PINs in roots, especially for PIN2 that underwent rapid degradation [68]. *MAB1* encodes a mitochondrial E1ß subunit of the pyruvate dehydrogenase complex in the TCA cycle. The amounts of PIN1 and PIN2 at the PM were reduced in *mab1* in a transcriptional behavior, likely due to the reduction of PIN endocytic recycling and acceleration of PIN vacuolar sorting [69]. Loss-of-function and dominant-negative (DN) mutations in ROP3 (Rho-related GTPase of plants 3) also cause reduced protein levels of PIN1-GFP and PIN3-GFP, which was inhibited by MG132 treatment.

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