In plants, the controlled absorption of soil nutrients by root epidermal cells is critical for growth and development. IRON-REGULATED TRANSPORTER 1 (IRT1) is the main root transporter taking up iron from the soil and is also the main entry route in plants for potentially toxic metals such as manganese, zinc, cobalt, and cadmium. Previous work demonstrated that the IRT1 protein localizes to early endosomes/trans-Golgi network (EE/TGN) and is constitutively endocytosed through a monoubiquitin- and clathrin-dependent mechanism. Here, we show that the availability of secondary non-iron metal substrates of IRT1 (Zn, Mn, and Co) controls the localization of IRT1 between the outer polar domain of the plasma membrane and EE/TGN in root epidermal cells. We also identify FYVE1, a phosphatidylinositol-3-phosphate-binding protein recruited to late endosomes, as an important regulator of IRT1-dependent metal transport and metal homeostasis in plants. FYVE1 controls IRT1 recycling to the plasma membrane and impacts the polar delivery of this transporter to the outer plasma membrane domain. This work establishes a functional link between the dynamics and the lateral polarity of IRT1 and the transport of its substrates, and identifies a molecular mechanism driving polar localization of a cell surface protein in plants.

Arabidopsis | endocytosis | nutrition | radial transport | PI3P

Iron is an essential element for virtually all organisms because it plays critical roles in life-sustaining processes (1). Iron’s ability to gain and lose electrons makes iron a cofactor of choice for enzymes involved in a variety of oxidation-reduction reactions, such as photosynthesis, respiration, hormone synthesis, and DNA synthesis. This essential role of iron is highlighted by the severe disorders triggered by iron deficiency, including anemia in mammals or chlorosis in plants (1). Although abundant in nature, iron is often available in limited amounts to plants because it is mostly found in rather insoluble Fe(III) complexes in soils (1). The IRON-REGULATED TRANSPORTER 1 (IRT1) root iron transporter from the model plant Arabidopsis thaliana takes up iron from the soil upon iron deficiency (2). IRT1 is a major player in the regulation of plant iron homeostasis, as attested by the severe chlorosis and lethality of an irt1-1 knockout mutant (2). Consistently, IRT1 gene is highly expressed in iron-starved root epidermal cells that face the rhizosphere (2). The resultant IRT1-dependent iron absorption allows proper growth and development under iron-limited conditions.

Despite its absolute requirement, iron reacts in cells with oxygen and generates noxious reactive oxygen species that are deleterious for plant growth and development (3). Cellular and whole-organism iron homeostasis must, therefore, be strictly balanced. Moreover, IR1 also participates in the absorption of zinc, manganese, cobalt, and industrial pollutants such as cadmium and nickel (4–8). As such, IRT1 is the main entry route for such potentially toxic metals in iron-starved plants and in the food chain. Intricate regulatory networks control plant responses to low iron conditions and, more specifically, IRT1 gene expression. Several transcription factors directly binding to the IRT1 promoter in root epidermal cells have been identified and control its inducibility by low iron conditions (9–12). Other pathways including the cytokinin-mediated root growth control and the stress hormone ethylene impinge on iron uptake by converging at the level of the IRT1 promoter (13, 14). The integration of these regulatory networks aims at providing enough iron to sustain growth and avoid detrimental effects of iron overload.

A posttranslational control of IRT1 protein by ubiquitination was identified (15, 16). IRT1 protein was shown to localize to early endosomes/trans-Golgi network compartments (EE/TGN) as a result of monoubiquitin- and clathrin-dependent endocytosis and is targeted to the vacuole for degradation via late endosomes (LE) (15). The ubiquitin-mediated endocytosis of IRT1 is mediated by the ID1/FRING E3 ligase (17). IRT1 localization and ubiquitination, however, appeared to be unaffected by the availability of its primary substrate iron (15), raising the question of the biological relevance of such posttranslational control of IRT1.

In the present study, we show that the availability in secondary non-iron metal substrates of IRT1 (i.e., Zn, Mn, and Co) controls its localization between the outer polar domain of the plasma membrane and EE/TGN in root epidermal cells. We identified a previously uncharacterized phosphatidylinositol-3-phosphate (PI3P)-binding protein recruited to LE, FYVE1, as an important regulator of IRT1-dependent metal transport and metal homeostasis. FYVE1 controls IRT1 recycling to the plasma membrane and impacts the polar delivery of this transporter to the outer plasma membrane domain. This work establishes a functional link between the dynamics and the lateral polarity of IRT1 and the transport of its substrates, and identifies a molecular mechanism driving polar localization of a cell surface protein in plants.

Significance

Plants take up iron from the soil by using a broad spectrum transporter named IRON-REGULATED TRANSPORTER 1 (IRT1). IRT1 mediates influx of potentially toxic elements such as manganese, zinc, cobalt, and cadmium. We uncovered that the localization at the cell surface of IRT1 is directly controlled by its secondary toxic substrates. When these metals are found at low levels in soils, IRT1 is located at the plasma membrane in a polar fashion facing the soil. We identified a lipid-binding protein recruited to endosomes that controls IRT1’s dynamics and polarity, and plays an important role in the radial transport of metals. Altogether, our work points to an unexpected mode of radial transport of iron toward vascular tissues involving efflux transporters.
link between the dynamics and the lateral polarity of IRT1 and the transport of its substrates, and identifies a molecular mechanism driving outer polar localization of a cell surface protein in plants.

Results and Discussion

The root iron transporter IRT1 undergoes Ub-dependent endocytosis, although this process is not regulated by the availability in iron, its primary substrate (15). To get further insight into the mechanisms driving IRT1 localization in EE/TGN, we tested the influence of the secondary metal substrates of IRT1 by immunolocalization using anti-IRT1-specific antibodies on plants constitutively expressing IRT1. Although IRT1 localized under standard conditions to intracellular vesicles that we previously characterized as EE/TGN (15), depletion of non-iron metal substrates of IRT1 led to its accumulation at the cell surface of root hairs (Fig. 1L). The EE/TGN identity was not altered by non-iron metal deficiency because known EE/TGN markers failed to relocalize to the cell surface under such growth conditions in differentiates root cells where IRT1 is expressed (Fig. S1A) (18, 19). These observations highlight the ability of potentially toxic non-iron metals transported by IRT1 to specifically trigger its intracellular dynamics between the cell surface and EE/TGN. The nonubiquitinatable IRT1K154RK179R was found at the cell surface in the presence of metals, as reported (15), providing genetic evidence that the response to the secondary substrates of IRT1 is likely mediated by Ub-mediated endocytosis (Fig. 1L). Metal-dependent endocytosis appears as a protective mechanism to limit the absorption of the secondary substrates of IRT1. Indeed, depletion of Mn, for example, from the medium alleviates the deleterious consequences of IRT1K154RK179R expression (Fig. 1B). Under iron limitation where IRT1 is strongly expressed, non-iron metals are readily available for transport by IRT1 and are heavily accumulated (2). In contrast, iron is not efficiently taken up because of its low level and the necessity of reduction by the FRO2 reductase whose activity is limiting for iron transport (20). Taken together, these observations point to the existence of multiple layers of regulation by metals for IRT1 gene expression. Iron indeed controls IRT1 transcription, whereas its secondary non-iron metal substrates act at the posttranscriptional level, as observed for Zn (21), and at the posttranslational level on the dynamics of IRT1 protein.

We also monitored IRT1 localization in response to metals in root epidermal cells. Interestingly, IRT1 accumulated under metal-depleted conditions at the outer polar domain of the plasma membrane facing the rhizosphere (Fig. 1C). Quantification of IRT1 fluorescence, represented as the fluorescence profile across root epidermal cells (Fig. S1B), show a clear enrichment of IRT1 in the outer plasma membrane domain. Lateral polarity in plant roots was described first in rice for the As/Si transporters Lsi1, Lsi2 (22), and in Arabidopsis for the boron transporter BOR4 (23). Then an increasing number of transporters were demonstrated to be laterally polarized in Arabidopsis roots such PDR8/PEX3, BOR1, NIP5;1, PIS1/PDR9/ABCG37, and NTA2.4 (24–28). However, the molecular mechanisms controlling lateral polarity are still unclear. To shed light on the mechanisms controlling IRT1 metal-dependent dynamics and its localization at the outer plasma membrane domain of root epidermal cells, we investigated the localization of the nonubiquitinatable IRT1K154RK179R mutant version. IRT1K154RK179R localized to the outer polar domain in the presence of metals, similar to what is observed with wild-type IRT1 under non-iron metal depleted conditions (Fig. 1C and D and Fig. S1B). We demonstrated that IRT1 accumulates at the cell surface in root hair cells when clathrin-mediated endocytosis (CME) is impaired (15). Inhibition of CME in root epidermal cells, due to tamoxifen-inducible expression of a dominant-negative clathrin form, led to the redistribution of IRT1 at the outer plasma membrane domain (Fig. 1C and D and Fig. S1B). The same DN-HUB1 line prevented the accumulation of the BR11 steroid hormone receptor in endosomal aggregates triggered by the fungal toxin brefeldin A (BFA) upon induction, ensuring that DN-HUB1 effectively inhibited CME (Fig. S1C). These results indicate that the presence of IRT1 in the outer plasma membrane polar domain is established independently of IRT1 ubiquitination and CME.
To identify factors involved in the control of IRT1 localization, we performed a yeast two-hybrid screen by using the hydrophilic cytosolic loop of IRT1 as bait. This screen identified several clones of an uncharacterized FYVE domain-containing protein named FYVE1 (Fig. 2A) (29). Expression analyses confirmed that FYVE1 transcripts are found in roots, similar to IRT1, although not regulated by iron starvation (Fig. S2A). To further confirm the protein–protein interaction between FYVE1 and IRT1, we first generated transgenic plants expressing FYVE1-mCitrine in the wild-type background. Using anti-GFP antibodies, FYVE1-mCitrine was immunoprecipitated from wild-type or FYVE1-mCitrine iron-deficient plants where IRT1 is expressed. The presence of IRT1 was only observed in immunoprecipitates from FYVE1-mCitrine (Fig. 2B), attesting that both proteins are able to interact in vivo.

FYVE domains have been reported to recognize PI3P, leading to recruitment of FYVE domain-containing proteins to EE and LE (30–34). To assess whether FYVE1 binds to PI3P, we performed lipid overlay analyses by using in vitro-transcribed/translated FYVE1-FLAG. FYVE1 interacted with PI3P, in agreement with previous reports on FYVE domain-containing proteins, and other acidic phospholipids, although to a lesser extent (Fig. 2C). Confocal microscopy imaging of FYVE1-mCitrine showed that FYVE1 protein is found in the nucleus, the cytoplasm, and in intracellular vesicles that may correspond to LE in root tip cells (Fig. S2B) and in differentiated root cells (Fig. 2D), although more difficult to visualize because of the large central vacuole. We further investigated FYVE1 localization in differentiated epidermal cells where IRT1 is expressed, and in root tip cells because they allow easy visualization of intracellular compartments, are readily accessible to drug and dye treatments, and have been extensively characterized (35). The recruitment of FYVE1 to LE was confirmed by its sensitivity to the inhibitor of PI3 kinase Wortmannin, which creates homotypic fusion and swelling of LE (Fig. 2E and Fig. S2C) (36). Consistently, FYVE1-mCitrine showed colocalization with the LE marker RabF2a (Fig. 2F and H and Fig. S2D and F) and the PI3P-recruited and LE-localized 2xFYVEHRS (Fig. 2G and H and Fig. S2E and F). Altogether those results demonstrate that IRT1 interacts with the LE-recruited and PI3P-binding protein FYVE1.

To functionally characterize FYVE1, we isolated the publicly available pSt18264 RIKEN insertion line, hereafter named fyve1-1. This line carries the Ds transposon in the first exon of the FYVE1 gene. We repeatedly failed to identify homozygous fyve1-1 knockout mutants in the progeny of fyve1-1 heterozygous transposon insertion lines, suggesting that the corresponding mutation is likely lethal at the homozygous state. Consistently, fyve1-1 segregating mutants produced approximately 26% of seeds that failed to germinate, corresponding to the ratio expected for plants segregating a recessive mutation impairing germination (Table 1). Genetic complementation of fyve1-1 was carried out by crossing heterozygous fyve1-1 mutant with a monoinsertional segregating line constitutively expressing Ubi10::FYVE1. The progeny now only showed 6.9% of seeds that failed to germinate, matching the expected segregation of 6.25% for plants carrying the homozygous fyve1-1 mutation and that do not possess transgenic FYVE1 (Table 1). Homozygous fyve-1 mutants carrying Ubi10::FYVE1 were also recovered in the progeny (Fig. S3A), further highlighting the genetic complementation of fyve1-1 by FYVE1. Overall, this genetic analysis clearly demonstrates that the loss-of-function mutation in the FYVE1 gene is the direct cause of fyve1-1 lethality. Because the onset of IRT1 expression is at 3 d after germination, we cannot use fyve1-1 to address the biological role of FYVE1 in iron homeostasis. We therefore generated transgenic plants constitutively expressing FYVE1 under the control of the strong 35S promoter, leading to overaccumulation of FYVE1 transcripts in transgenic plants (Fig. S3B). Such plants showed no phenotype when grown in the presence of iron in the medium (Fig. 3A and B). When grown on iron-depleted media, 35S::FYVE1 plants displayed shorter roots than wild type (Fig. 3A and B), a hallmark of iron-deficient plants. The hypersensitivity of 35S::FYVE1 plants to low iron conditions is abolished when plants are grown on medium containing Wortmannin (Fig. S3C), indicating that PI3P is necessary for the role of FYVE1 in iron homeostasis. Plants overexpressing the well-established PI3P-binding 2xFYVE_HRS reporter showed wild-type sensitivity.
to low iron, attesting the specific involvement of FYVE1 in plant responses to low iron (Fig. 3 A and B) (34). To further evaluate the biological role of FYVE1 in metal homeostasis, we determined the metal content of both wild-type and 35S::FYVE1 plants by Inductively-Coupled Plasma Mass Spectrometry (ICP-MS). Under metal-replete conditions, both wild-type and 35S::FYVE1 plants showed comparable metal accumulation profile (Fig. 3C). When grown in the absence of iron, however, 35S::FYVE1 roots accumulated slightly less Fe and showed significantly reduced levels of non-iron IRT1 substrates. As a control, we monitored accumulation of boron that is not transported by IRT1 and observed no difference between the two genotypes (Fig. 3C).

The fact that 35S::FYVE1 showed hypersensitivity to low iron growth conditions and reduced accumulation of metals transported by IRT1 prompted us to investigate IRT1 expression in both wild-type and 35S::FYVE1 plants. FYVE1-overexpressing plants accumulated wild-type levels of IRT1 protein (Fig. 4A), indicating that the phenotypes displayed by 35S::FYVE1 plants are not explained by lower accumulation of IRT1 transporter. We then analyzed IRT1 protein localization by immunolocalization to highlight possible defects in IRT1 localization. Wild-type plants grown in the absence of iron showed strong accumulation of IRT1 in EE/TGN, as reported (15), whereas 35S::FYVE1/IRT1 fusion protein in the recycling of IRT1 from endosomal compartments.

Table 1. fyve1-1 germination phenotype and genetic complementation

<table>
<thead>
<tr>
<th>Parental genotype</th>
<th>Germinated, %</th>
<th>Ungerninated, %</th>
<th>n</th>
<th>χ²</th>
<th>P value</th>
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<tr>
<td>WT</td>
<td>97.7</td>
<td>2.3</td>
<td>176</td>
<td></td>
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<tr>
<td>fyve1/FYVE1</td>
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<td>29.1</td>
<td>617</td>
<td>2.628</td>
<td>0.019*</td>
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<tr>
<td>fyve1/FYVE1 Ub10::FYVE1 +/+</td>
<td>91.8</td>
<td>9.2</td>
<td>455</td>
<td>2.634</td>
<td>0.011*</td>
</tr>
<tr>
<td>fyve1/FYVE1 (corrected)</td>
<td>73.2</td>
<td>26.8</td>
<td>617</td>
<td>0.290</td>
<td>0.373</td>
</tr>
<tr>
<td>fyve1/FYVE1 Ub10::FYVE1 +/+ (corrected)</td>
<td>93.1</td>
<td>6.9</td>
<td>455</td>
<td>0.037</td>
<td>0.729</td>
</tr>
</tbody>
</table>

Segregation of the progeny phenotypes (germinated:ungerminated) were corrected for the germination defect of wild-type (WT) Nossen ecotype and evaluated with the χ² goodness-of-fit test by using 3:1 segregation as a null hypothesis for fyve1-1 and a 1:3 segregation as a null hypothesis for fyve1 Ub10::FYVE1. χ² values (χ²) and corresponding P values are indicated. *Significant difference (P < 0.05).

Fig. 3. FYVE1 overexpression leads to iron deficiency and impaired transport of IRT1 substrates. (A) Phenotype of wild-type (WT), two independent 35S:FYVE1, and two independent 35S::2xFYVEHRS transgenic lines grown 5 d in +Fe (Upper) or in -Fe (Lower). (B) Root length of 5-d-old wild-type (WT), 35S:FYVE1, and 35S::2xFYVEHRS grown with or without Fe. Results are presented as mean ± SD (n = 20). Statistical differences were calculated by one-way ANOVA. Different letters indicate means that were statistically different by Tukey’s multiple testing method (P < 0.05). (C) Metal content determined by ICP-MS on roots of 7-d-old plants grown with or without Fe. Results are presented as mean ± SD from three to four batches of 30 seedlings. Statistical differences were calculated by one-way ANOVA. Different letters indicate means that were statistically different by Tukey’s multiple testing method (P < 0.05) for genotypes within a given growth condition (+Fe or -Fe).
Defects within the root and, thus, impaired metal accumulation. The loss of polarity would result in IRT1 working epidermal cells using efflux transporters rather than the symplasmic route. Whether iron and metal exit from epidermal cells will have to be addressed in the future.

In plants, the necessity of a functional interface between the root and the soil is obvious, but virtually nothing is defined. Here, we demonstrated that the localization of the IRT1 root iron transporter is dynamically controlled between the EE/TGN and the cell surface by its potentially toxic secondary substrates to avoid non-iron metal toxicity. In addition, we identified an endosomal-recruited protein controlling not only the localization but also the polarity of IRT1, and, thus, establishing the functional link between lateral polarity of a transporter and transport of its substrate. Finally, our work also opens the door to a better understanding on the establishment of polarity for other proteins targeted to the outer plasma membrane domain and acting as nutrient and hormone transporters or mediators of pathogen defense (22–25, 27, 28).

**Methods**

**Materials and Growth Conditions.** Wild-type and the various transgenic lines used in this study were grown in sterile conditions on vertical plates at 21°C with 16-h light/8-h dark cycles, as described (14). For expression analyses, plants were cultivated in the conditions described above for 7 d and then transferred on iron-sufficient (50 μM Fe-EDTA) or iron-deficient (300 μM Ferrozine [3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine sulfonate], a strong iron chelator) medium for an additional 3 d. For immunolocalization studies, iron starvation was applied by directly germinating seeds on half-strength Murashige and Skoog (MS) medium lacking exogenous iron to preserve root integrity. All reagents were purchased from Sigma-Aldrich.

The *irt1-1, irt1-1/35S::IRT1, irt1-1/35S::IRT1K154RK179R* dominant negative clathrin hub DN-HU1B1, RabF2a-mCherry, 2xFYVEHRS-mCherry, 2xFYVEHRS-RBI1-mCitrine, VHA1-GFP, and VT112-YPF lines were described in previous studies (18, 19, 34, 35, 37). The *fye1-1* (77-3D) plant (*fye1-1* Nossen ecotype) was isolated from the RIKEN collection. Genotyping of *fye1-1* was carried out with the primers listed in SI Methods.

**Imaging.** FM4-64 (Invitrogen) was applied at a concentration of 5 μM; Cy3-cloheximide (Sigma-Aldrich) was applied at a concentration of 100 μM for 1 h before treatment with BFA; BFA and Wortmannin (Sigma-Aldrich) were applied at a concentration of 50 μM and 33 μM for 1 h in liquid medium, respectively.

Imaging was performed on an inverted Leica SP2 and Zeiss 700 confocal microscopes. The percentage of FYVE1-mCitrine dotted structures showing overlap with RabF2a-mCherry or 2xFYVEHRS-mCherry was manually determined. Induction of DN-HU1B1 was performed with 2 μM 2-hydroxytamoxifen for 24 h. For polarity profiles, epidermal cell width was quantified and the moving average was applied to the scatterplot. The ratios between PM_in and fluorescence intensity determined across cells with ImageJ. The overlap with RabF2a-mCherry or 2xFYVEHRS-mCherry was manually determined. The *fye1-1* plant (*fye1-1* Nossen ecotype) was isolated from the RIKEN collection. Genotyping of *fye1-1* was carried out with the primers listed in SI Methods.

The FYVE1 ORF was cloned into pCHF3 binary vector under the control of 35S promoter, or recombined by multisite Gateway technology to generate *ubi10::FYVE1-mCitrine* in the pB7m34GW binary vector. For lipid binding assays, FYVE1 was recombined in pTNT GW-HF, a gateway compatible in vitro transcription/translation vector carrying a FLAG tag (55).
addition were analyzed by using ImageJ. To better visualize the endosomas, images were treated with the Difference of Gaussian filter, and the number of FM4-64-positive endosomes in a cell was quantified on the treated images by using the 3D Object Counter plugin of ImageJ.

**Elemental Analyses.** Tissues were digested for 5 min with 2 mM CaSO₄ and 10 mM EDTA and rinsed for 2 d with deionized water. Samples were dried at 80 °C for 2 d. For mineralization, tissues were digested completely (1–3 h) in 70% (vol/vol) HNO₃ at 120 °C. Elemental analyses were performed by ICP-MS.


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Supporting Information

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SI Methods

RNA Extraction and Real-Time Quantitative PCR. Total RNA was extracted by using TRIzol reagent (Invitrogen) and purified by using the RNeasy MinElute Cleanup Kit (Qiagen) after DNase treatment (Qiagen). The integrity of DNA-free RNA was verified by agarose gel electrophoresis, and an equal amount of total RNA (2 μg) was used for reverse transcription with anchored oligo (dT23). Real-time PCR was performed exactly as described (1), using gene-specific and control primers. The experiments were done in three biological replicates, with each containing two technical replicates.

Protein Extraction and Western Blot Analysis. Western blot analyses of IRON-REGULATED TRANSPORTER 1 (IRT1) were performed on total proteins exactly as described (1, 2). Immunodetection of mCitrine was performed by using the same protocol, but using the HRP-coupled GFP antibodies (Miltenyi Biotech).

Immunoprecipitation. Roots of plants cultivated in the conditions described above were subjected to immunoprecipitation as described (3), and following manufacturer’s instruction (Miltenyi Biotech).

Yeast Two-Hybrid Screen. A fragment of the IRT1 cDNA, encoding the cytosolic loop of IRT1 (amino acids 146–194), was cloned into the pGBK7 vector (Clontech) and transformed in the AH109 yeast strain, as described (2). The resulting yeast cells were then transformed with plasmid DNAs prepared with roots and shoots of 4-wk-old plants. Approximately 10⁶ transformants were screened for activation of the HIS3 and LacZ reporters. Positive clones were sequenced, and interesting candidates in which GAL4AD was in frame with the corresponding cDNA were rescued. Candidate clones isolated from the screen were then individually retransformed in yeast cells expressing GALDB alone or the GAL4DB-IRT1 prey fusion for confirmation.

Lipid Overlay Assays. Lipid binding assays were performed by using PIP Strips (Echelon). PIP strips were blocked in 1× TBS, 0.1% Tween 20, and 5% fatty acid-free BSA and then incubated overnight at 4 °C in vitro transcribed/translated FYVE1-FLAG by using reticulocytes (Promega). Next, the PIP strips were washed with 1× TBS and 0.1% Tween 20, incubated with anti-FLAG antibodies (1:2,000 in blocking solution) for 1 h at room temperature, washed with 1× TBS and 0.1% Tween 20, and then incubated with anti-rabbit HRP secondary antibodies (1:1,000) for 1 h at room temperature. Finally, the PIP strips were washed with 1× TBS and 0.1% Tween 20 before developing with enhanced chemiluminescent reagent.

Immunolocalization. Whole-mount immunolocalization experiments were performed as described (2).

List of Primers Used in This Study. Construct pCHF3-35S::FYVE1.
- FYVE1 F: 5′-GGGAGCTCATGCAACAGGGAGATTACAAATTCG-3′
- FYVE1 R: 5′-GCGGATCCTCAATGTGCGCTAACGAGGAAAGG-3′

Construct pDONR221-FYVE1.
- FYVE1attB1: 5′-GGGGACAAGTTGTGACAAAAAAGCAGGCTGGGCCATGGACAGGAGATTACAATTCG-3′
- FYVE1attB2: 5′-GGGGACCACTTTGTACAAAAAAGCAGGCTGGGCCATGCAACAGGGA-3′

qRT-PCR.
- qFYVE1 F: 5′-CGGTTGGACTTCTAAATGC-3′
- qFYVE1 R: 5′-CGGGTAGGAAGATCAATGTGC-3′
- IRT1 F: 5′-CGGTTGGACTTCTAAATGC-3′
- IRT1 R: 5′-CGATAATCGACATTCCACCG-3′
- Clathrin F: 5′-AGCATACACTGCGTGCAAAG-3′
- Clathrin R: 5′-TCGCTGTGTCATATCTC-3′

- EI511: 5′-GCGACATCACTAAACCC-3′
- EI512: 5′-AACCCACCAACATAAGAAC-3′

T-DNA–specific primer.
- Ds5-2a: 5′-TCCGTTCCGTTTTCGTTTT-3′

Ubi10::FYVE1–specific primers.
- CK150: 5′-GGAGCATCCATTAGCTTGTGCT-3′
- EI2: 5′-GGGAGACCACCTTTGTGATACAGAAAGCTGGGTCT-3′

Fig. S1. Metal-dependent dynamics and polar localization of IRT1. (A) Confocal microscopy imaging of the EE/TGN markers VTI12-YFP and VHAa1-GFP in differentiated root cells of plants grown in control or metal-depleted conditions. (B) Quantification of polarity profiles across epidermal cells (Fig. 1C). Data represent the best fitted curve corresponding to independent immunofluorescence experiments \((n = 10)\). (C) Confocal imaging of BRI1-mCitrine and BRI1-mCitrine/DN-HUB1-RFP. DN-HUB1 is induced by tamoxifen treatment before brefeldin A application. The overlay between the yellow (BRI1-mCitrine) and red (DN-HUB1-RFP) channels is shown. (Scale bars: A, 10 μm; C, 30 μm.)
Fig. S2. Characterization of FYVE1 localization. (A) Quantitative RT-PCR analyses monitoring FYVE1 (Left) and IRT1 (Right) expression in roots of wild-type plants. Experiments were performed by using 10-d-old plants transferred for 3 d in iron-sufficient (+Fe) or iron-deficient (-Fe) conditions. Results are presented as mean ± SD from three to four batches of 30 seedlings. (B) Confocal microscopy imaging of FYVE1-mCitrine plants in root tip cells. Inset is shown on Right. (C) Sensitivity of FYVE1-mCitrine trafficking to Wortmannin (Wm) in root tip cells. Arrows show an example of a Wortmannin compartment. Inset is shown on Right. (D and E) Colocalization of FYVE-mCitrine with the late endosomal marker RabF2a-mCherry (D) and 2xFYVE_HRS-mCherry (E) in root tip cells. (F) Quantification of FYVE1 colocalization with the late endosomal markers RabF2a and 2xFYVE_HRS. Colocalization of punctate structures was quantified in 10 cells from the F1 progeny from crosses between parental lines FYVE1-mCitrine and marker lines RabF2a and 2xFYVE_HRS. Results are presented as mean ± SE. (Scale bars: B, 25 μm; C, 10 μm; and D and E, 5 μm.)
Fig. S3. Genetic characterization of FYVE1. (A) Phenotype of 3-wk-old wild type (WT), heterozygous fyve1/FYVE1, and homozygous fyve1/fyve1 carrying Ubi10::FYVE1 transgene (Upper). The genotypes of the photographed plants were verified by PCR-based genotyping (Lower). Genotyping was performed for the endogenous gene (G), for the T-DNA (T), and for the transgene (Tr). (B) Quantitative RT-PCR analyses monitoring FYVE1 (Left) and IRT1 (Right) expression in roots of wild-type and two 35S::FYVE1 independent transgenic lines. Experiments were performed by using 10-d-old plants grown in MS/2. Results are presented as mean ± SD from three to four batches of 30 seedlings. (C) Root length of 7-d-old wild-type (WT) and two independent transgenic lines of 35S::FYVE1 grown in +Fe or –Fe conditions with various concentrations of Wortmannin. Results are presented as mean ± SD (n ≥ 8). Different letters indicate means that were statistically different by one-way ANOVA and Tukey’s multiple testing method (P < 0.05). (D) FM4-64 internalization assays. Seven-day-old wild-type and 35S::FYVE1 plants were treated with FM4-64, rinsed, and imaged every 5 min after treatment. Representative images of FM4-64 signals obtained in root tip cells (Upper) and differentiated root cells (Lower) after 25 min are shown. (Scale bar: 10 μm.) (E) Quantification of FM4-64 internalization on root tip cells (Upper) and differentiated root cells (Lower). Results are represented as mean ± SE (n = 10).