

AtEHDs, novel Arabidopsis EH-domain-containing proteins involved in endocytosis

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Summary

Endocytosis is an essential process by which the eukaryotic cell internalizes exogenous material. Studies in yeast and mammalian cells have revealed that endocytosis is a complex molecular process depending on regulated interactions between a variety of proteins and lipids through specific modules. One such module is the Eps15 homology (EH) domain, a conserved modular protein-interaction domain found in several endocytic proteins. The EH-domain-containing proteins function as regulators of endocytosis through their ability to interact with other proteins involved in this process. Here we describe the isolation and characterization of two Arabidopsis EH-domain-containing proteins (AtEHD1 and AtEHD2). We show that the two proteins are involved in endocytosis in plant systems and demonstrate that the Arabidopsis EHD proteins function similarly to mammalian EHDs. Similarly to hEHD2, over-expression of AtEHD2 has an inhibitory effect on endocytosis. While transgenic plants over-expressing AtEHD1 had no detectable phenotype, downregulation of AtEHD1 caused retardation of entry of endocytosed material into plant cells.

Keywords: Arabidopsis, EHD1, Eps15 homology domain, endocytosis.

Introduction

Endocytosis is the process by which cells take up extracellular substances and/or internalize plasma membrane proteins for transport to endosomes (Murphy *et al.*, 2005). Mammalian endocytosis regulates many processes, such as homeostasis, nutrient uptake, cell polarity, retrieval of exocytosed vesicle components, downregulation of signaling receptors, localization/abundance of membrane transporters and cell-to-cell signaling (Le Roy and Wrana, 2005; Mukherjee *et al.*, 1997; Samaj *et al.*, 2005). From the early or sorting endosomes, plasma membrane proteins are either targeted to the lysosome for degradation or recycled back to the cell surface (Le Roy and Wrana, 2005; Mukherjee *et al.*, 1997; Samaj *et al.*, 2005). There are several forms of endocytosis: clathrin-mediated endocytosis; caveolin-mediated endocytosis and clathrin- and caveolin-independent endocytosis (Conner and Schmid, 2003).

Endocytosis depends on a large number of protein-protein interactions mediated by specific modules. One such module is the EH (Eps15 homology) domain (EHD) first identified in Eps15 (Carbone *et al.*, 1997; Wong *et al.*, 1995).

The EH domain structure generally consists of two EF-hands and a helix-loop-helix structure that binds calcium (or a pseudo EF-hand), connected by an anti-parallel beta-sheet (de Beer *et al.*, 2000; Di Fiore *et al.*, 1997; Santolini *et al.*, 1999). The majority of the EH-binding proteins contain an NPF (Asn-Pro-Phe) motif. Thirty-five EH-containing proteins have been identified so far in different species, with 11 proteins identified in humans, among them EHD-containing proteins 1–4 (EHD1–4), Eps15 and Intersectin 1–2 (Polo *et al.*, 2003). The EH-containing and EH-binding proteins together constitute the EH-network (Polo *et al.*, 2003).

The EHD family contains four members in mouse and human designated EHD1–4. One homolog was found in *Drosophila* and *Caenorhabditis elegans* named PAST-1 and RME-1, respectively. All members share a similar structure: an N-terminal domain with a nucleotide-binding motif, GxxxxGKTxxxxxx (P-loop), DxxG and NKxD, a central coiled-coil region and a C-terminal EHD, containing an EF Ca²⁺-binding motif. A nuclear localization signal (NLS) was also predicted for all the family members. The crystal

structure of hEHD2 was recently solved, providing evidence for nucleotide-dependent membrane remodeling (Daumke *et al.*, 2007). Additionally, modeling of the EH domain in hEHD1 indicates that the binding pocket in hEHD1 is narrower and not as deep as that of the Eps15 EH-2 domain (Kieken *et al.*, 2007). Although the four human EHD proteins share high homology at the protein level and share similar domains, they appear to mediate different roles in the intracellular trafficking machinery (Blume *et al.*, 2007; Galperin *et al.*, 2002; George *et al.*, 2007; Gokool *et al.*, 2007; Guilherme *et al.*, 2004; Naslavsky and Caplan, 2005; Rapaport *et al.*, 2006).

The first clue for the involvement of the EHD family in trafficking and endocytosis was revealed when a genetic screen for endocytosis genes identified *RME-1* as a key player in transport regulation through the endocytic recycling compartment (ERC); however, there was no detectable effect on internalization (Lin *et al.*, 2001). The protein is localized mainly to the ERC. It plays an important role in ERC morphology, and recycling regulation from the ERC to the plasma membrane has been established in *C. elegans* (Grant *et al.*, 2001) and mammalian cells (Galperin *et al.*, 2002; Rapaport *et al.*, 2006).

Endocytosis has not been as extensively researched in plants as in mammals, but the field is rapidly evolving. It has been demonstrated in several systems that plant proteins can internalize from the plasma membrane in turgid cells (Geldner *et al.*, 2001; Grebe *et al.*, 2003; Meckel *et al.*, 2004; Murphy *et al.*, 2005; Ron and Avni, 2004; Russinova *et al.*, 2004; Samaj *et al.*, 2005).

Plant endocytic compartments are not well characterized and the term endosome is often employed generally for compartments containing endocytosed material. Styryl dyes such as FM-4-64 have been used to study the localization of vesicles which are putative endosomes (Bolte *et al.*, 2004; Grebe *et al.*, 2003; Lam *et al.*, 2007; Ueda *et al.*, 2001). Structural studies indicated that the partially coated reticulum (PCR) is analogous to the early/recycling endosomes of mammals (Galway *et al.*, 1993). Two distinct classes of early endosomes have been identified in Arabidopsis. One comprises the endosomes in which Ara6 resides, and the other the endosomes to which Ara6 is not targeted (Ueda *et al.*, 2001). Early endosomes have also recently been characterized as SCAMP1-containing tubular-vesicular structures possessing clathrin coats and residing in the vicinity of the trans-Golgi network (TGN; Lam *et al.*, 2007). Molecule sorting occurs in the early endosomes, from which they are either recycled back to the plasma membrane or transported to the Golgi apparatus or to multi-vesicular bodies (MVBs, also known as late endosomes) (Battey *et al.*, 1999; Jurgens, 2004). The TGN was also found to be involved in early endocytic pathways in Arabidopsis (Dettmer *et al.*, 2006; Lam *et al.*, 2007). Pre-vacuolar compartments (PVCs) have been identified as MVBs in tobacco BY2 cells (Tse *et al.*, 2004). From the MVBs, the endocytosed material is targeted

to the vacuole for degradation. Recent studies conducted in plant systems have further elucidated possible functionalities of plant endocytic compartments and the flow of endocytosed material throughout plant cells (Geldner *et al.*, 2007; Lisboa *et al.*, 2008; Muller *et al.*, 2007; Robatzek, 2007; Silady *et al.*, 2008; Teh and Moore, 2007).

Clathrin-coated vesicles are most probably a major means of internalization in plant cells. Studies conducted recently have demonstrated that clathrin-dependent internalization occurs in plants. (Dhonukshe *et al.*, 2007; Lam *et al.*, 2007; Leborgne-Castel *et al.*, 2008; Perez-Gomez and Moore, 2007; Tahara *et al.*, 2007). Components that interact with the clathrin-coated vesicles and adaptor proteins, such as dynamins and proteins that contain an SH3 domain, occur in plants and are involved in endocytosis and vesicle trafficking (Kang *et al.*, 2003; Lam *et al.*, 2001).

Recently, Ortiz-Zapater *et al.* (2006) reported that the human transferrin receptor is functional in transgenic Arabidopsis protoplasts, where it mediates binding and internalization of its natural ligand transferrin and binds to the endogenous Arabidopsis medium adaptin subunit. This indicates a high level of functional conservation between mammalian and plant endocytic systems.

While plant endocytosis has become a center of attention in recent years, the plant proteins participating in endocytic processes need to be further characterized. This work describes the isolation and characterization of two Arabidopsis EHD-containing proteins (AtEHD1 and AtEHD2). We show here that the two proteins are involved in endocytosis in plant systems and demonstrate that the Arabidopsis EHD proteins function similarly to mammalian EHDs. Downregulation of *AtEHD1* causes delay in internalization. Furthermore, over-expression of *AtEHD2* has an inhibitory effect on endocytosis.

Results

Isolation and characterization of Arabidopsis EHDs

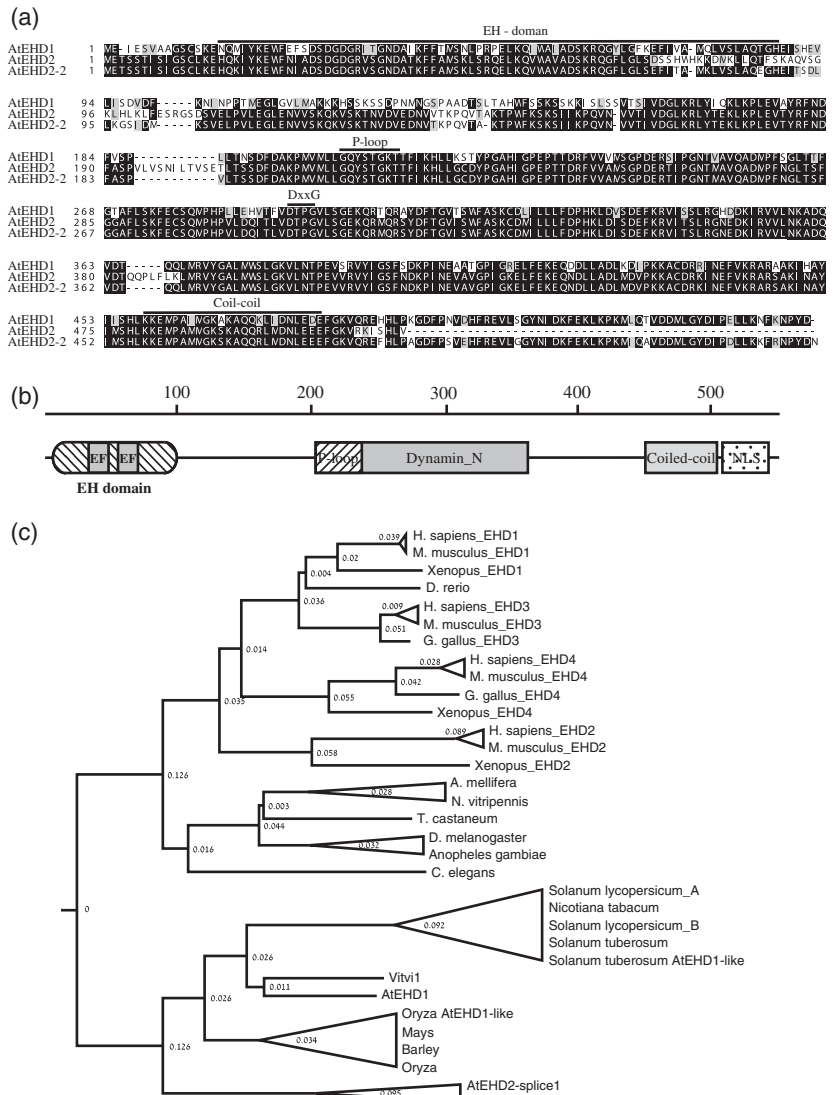
Using the BLAST (Altschul *et al.*, 1997) program we identified two open reading frames in the Arabidopsis genome (Figure 1) that show relatively high homology to each of the mammalian EHD proteins, and contain analogous domains to those present in mammalian EHDs. Searching the available plant sequence databases revealed that proteins homologous to Arabidopsis EHD exist in Solanaceae as well as in rice and maize. The Arabidopsis proteins showed 74% similarity to mammalian EHD1. The Arabidopsis EHD genes, termed *AtEHD1* (*At3g20290*) and *AtEHD2* (*At4g05520*), were isolated by PCR amplification on a total leaf cDNA template. *AtEHD1* encodes a predicted protein of 61 kDa with a predicted pI of 7, while *AtEHD2* encodes a predicted protein of 58 kDa with a predicted pI of 9. Both proteins contain an EH domain with two EF calcium-binding hands, a P-loop

Figure 1. Alignment and domain analysis of Arabidopsis Eps15 homology (EH) domain-containing proteins (AtEHDs).

(a) Multiple alignment of the predicted amino acid sequences of AtEHD1, AtEHD2 and AtEHD2-2. Accession numbers are as follows: AtEHD1 (At3g20290); AtEHD2 (At4g05520) and AtEHD2-2 (At4g05520). Identical amino acids are shaded in black, similar amino acids are shaded in light gray. Sequences were aligned using CLUSTALW (<http://www.ebi.ac.uk/clustalw>) and BoxShade software (http://www.ch.embnet.org/software/BOX_form.html).

(b) Schematic representation of the predicted structure of the Arabidopsis EHDs. The regions encoding the different protein domains are shown.

(c) Phylogenetic analysis of EHDs across kingdoms. The tree was constructed by the neighbor-joining method (Saitou and Nei, 1987). The presented tree was generated by FIGTREE® software (<http://tree.bio.ed.ac.uk/software/figtree>) using sequences aligned with CLUSTALW (<http://www.ebi.ac.uk/clustalw>). Numbers represent branch length.



(GxxxxGKS/T in general and in AtEHD1/2: GQYSTGKT, 100% conserved with the hEHD1 P-loop) and DxxG (DTPG in AtEHD1/2) with a predicted ATP/GTP-binding site, a bipartite NLS and a coiled-coil domain, as well as a Dynamin-N motif (Dynamin like GTPase domain). The NKxD sequence conserved in the mammalian proteins is absent in the plant gene products. The two Arabidopsis proteins share 80% similarity and 69% identity. The alignment and schematic domain representation of both proteins are presented in Figure 1. The major conserved regions in the mammalian EHD proteins are also present in the Arabidopsis proteins. The main difference between the plant and the mammalian proteins is the localization of the EH domain. In mammalian EHD proteins the EH is present at the C-terminus of the proteins while it is present at the N-terminus of the Arabidopsis EHD proteins. Though there are several proteins containing an N-terminal EHD in mammals and other organisms (Naslavsky and Caplan, 2005), based on domain

analysis and overall homology, AtEHD1 and AtEHD2 most resemble the mammalian EHD proteins. A phylogenetic tree of EHDs across kingdoms is presented in Figure 1(c). As expected, the various plant proteins included in the analysis are on a separate branch from the mammalian (and all other) EHD proteins. Interestingly, mammalian EHD2 and Arabidopsis EHD2 are on a separate branch compared with the other EHD proteins in the same species.

Analyzing the EST database revealed that the *AtEHD2* locus encodes an additional open reading frame as a result of a splice variation. We isolated this variant and called it *AtEHD2-2*. *AtEHD2-2* bears 89% homology to *AtEHD2* and 75% homology to *AtEHD1* at the protein level (Figure 1).

According to expression profiles available at Geneinvestigator (Zimmermann *et al.*, 2004) *AtEHD1*, *AtEHD2* and *AtEHD2-2* are ubiquitously expressed in all plant tissues, possibly hinting to the importance of their function. Semi-quantitative RT-PCR results show that *AtEHD1* has a

markedly higher mRNA expression level than *AtEHD2* and *AtEHD2-2* (data not shown).

Localization of AtEHD proteins in plant cells

The sub-cellular localization of AtEHD1 and AtEHD2 was examined in plant cells. Expression of AtEHD1-GFP and AtEHD2-GFP in *Nicotiana tabacum* revealed that both proteins are localized to the cytoplasm and the membrane (Figure 2a). AtEHD1 was also found to reside in vesicular structures, most likely endosomes, while AtEHD2 can also be found in the nucleus. Figure S1 shows the localization of AtEHD1-GFP in stable transgenic Arabidopsis plants. Despite several attempts, we were not able to obtain transgenic AtEHD2-GFP Arabidopsis plants.

In order to gain additional insight into the sub-cellular localization of AtEHD1 and AtEHD2, cell fractionation experiments were performed (Bar-Peled and Raikhel, 1997). Total cell lysates were prepared from transgenic as well as transiently transformed tobacco plants expressing AtEHD1 or AtEHD2 under the control of the 35S promoter. Various

treatments to extract the AtEHD proteins from the microsomal pellet were examined. Equivalent amounts of the microsomal protein pellet (p150) were resuspended in buffers as indicated in Figure 2(b). Both proteins were found in the cytoplasmic as well as the microsomal fractions of the lysate (Figure 2b). Furthermore, only buffers containing sarcosyl were able to efficiently extract the AtEHD proteins from the microsomal fraction, suggesting that the association of AtEHD proteins with plant microsomes and/or membranes is strong.

In order to further confirm the results suggesting that AtEHD1 and AtEHD2 localize to endosomal and/or membranous fractions, the styryl dye FM-4-64 was employed. FM-4-64 can only be observed when in a lipid environment. FM-4-64 was shown to enter plant cells via endocytic pathways, and is commonly used as an endocytic marker (Aniento and Robinson, 2005). FM-4-64 was recently used in a study aimed at elucidating plant endosomal sub-cellular organelle structures (Ueda *et al.*, 2001; Vida and Emr, 1995), as well as in other studies of plant endocytosis (Geldner *et al.*, 2003; Grebe *et al.*, 2003; Lam *et al.*, 2007; Murphy *et al.*, 2005; Russinova *et al.*, 2004).

Leaf epidermal cells of *Arabidopsis thaliana* cv. Columbia stably expressing AtEHD1-GFP fusion protein and *N. tabacum* cv. Samsun transiently expressing AtEHD1-GFP or AtEHD2-GFP fusion protein were injected with 5 μ M FM-4-64 with a needless syringe. Sixty minutes after injection, leaf sections were visualized under a laser-scanning confocal microscope. FM-4-64 appears to co-localize with AtEHD1 in the plasma membrane, as well as on dot-like structures which are positive for both AtEHD1-GFP expression and FM-4-64 fluorescence (Figure 3 and Figure S2), thereby confirming that AtEHD1 localizes to membranous endocytic vesicles (Ueda *et al.*, 2001). Moreover, FM-4-64 is co-localized with AtEHD2-GFP in leaf epidermal cells of *N. tabacum* cv. Samsun plants expressing AtEHD2-GFP (Figure 3). Thus, we conclude that both proteins are co-localized with membranous organs – AtEHD1 mostly with endosomes and AtEHD2 mostly with the plasma membrane. Figure S3 shows that AtEHD1-GFP is co-localized with FM-4-64 in the membrane and on dot-like structures in root cells of stable transgenic AtEHD1-GFP Arabidopsis seedlings.

To rule out a possible Golgi identity of the vesicular structures observed in plant cells expressing AtEHD proteins, we conducted co-expression studies with an mRFP fusion protein of the Golgi marker sialyl transferase (Brandizzi *et al.*, 2002) in plant cells. The AtEHDS did not co-localize with the Golgi marker (Figure 4), confirming that plant AtEHD proteins do not reside on Golgi bodies.

To confirm the localization of AtEHD1, the endosomal identity of the vesicular structures observed above (Figures 2 and 3, Figure S2) was also examined by co-localization of AtEHD1 with the plant endosomal markers Ara-6 (Grebe *et al.*, 2003; Ueda *et al.*, 2001) and FYVE (Heras

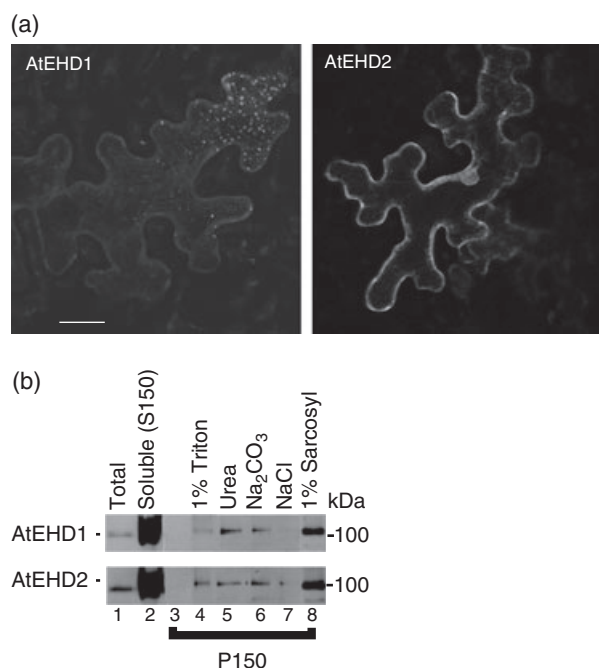


Figure 2. Localization of Arabidopsis Eps15 homology (EH) domain-containing protein 1 (AtEHD1) and AtEHD2 in plant cells.

(a) Tobacco plants transiently expressing AtEHD1-GFP (left) and AtEHD2-GFP (right). Leaf sections were visualized under a laser-scanning confocal microscope (Zeiss). Scale bar represents 10 μ m.

(b) Lysates of *Nicotiana tabacum* plants transiently expressing AtEHD1-GFP or AtEHD2-GFP were fractionated as described in Experimental procedures. Fractions (20 μ g per lane) were separated on 12% acrylamide SDS-PAGE and analyzed by immunoblotting using anti-GFP antibodies, as follows: lysis buffer alone (lane 3), lysis buffer containing 1% of the non-ionic detergent Triton X-100 (lane 4) or 2 M urea (lane 5), 0.1 M Na₂CO₃ (lane 6), lysis buffer supplemented with 1 M NaCl (lane 7) and lysis buffer containing 1% of the ionic detergent sarcosyl (lane 8).

Figure 3. Localization of the endocytic marker FM-4-64 in leaf tissue.

Arabidopsis leaves stably expressing Arabidopsis Eps15 homology (EH) domain-containing protein 1 (AtEHD1)-GFP (upper panel) or *Nicotiana tabacum* transiently expressing AtEHD2-GFP (lower panel) were injected with 5 μM FM-4-64. FM-4-64 and GFP fluorescence were visualized in leaf sections 120-min post-FM-4-64 injection under a laser-scanning confocal microscope (Zeiss). Scale bar represents 10 μm . Arrow heads indicate co-localized pixels.

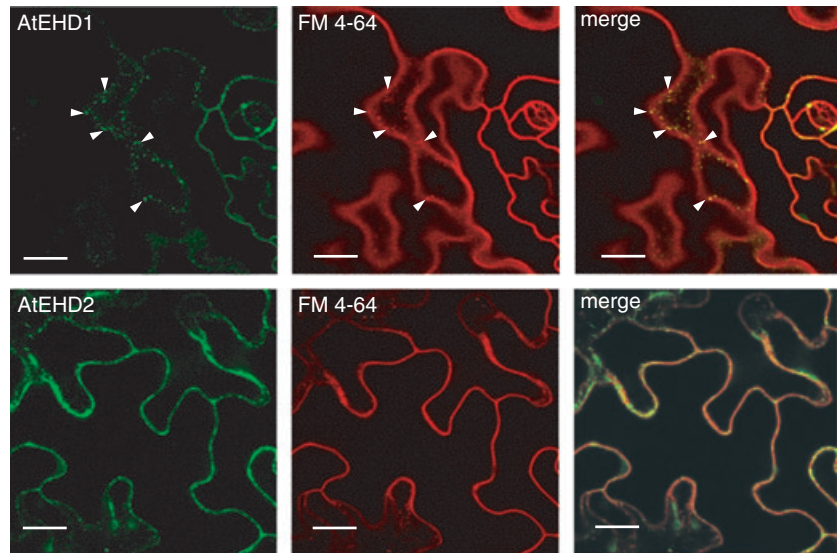
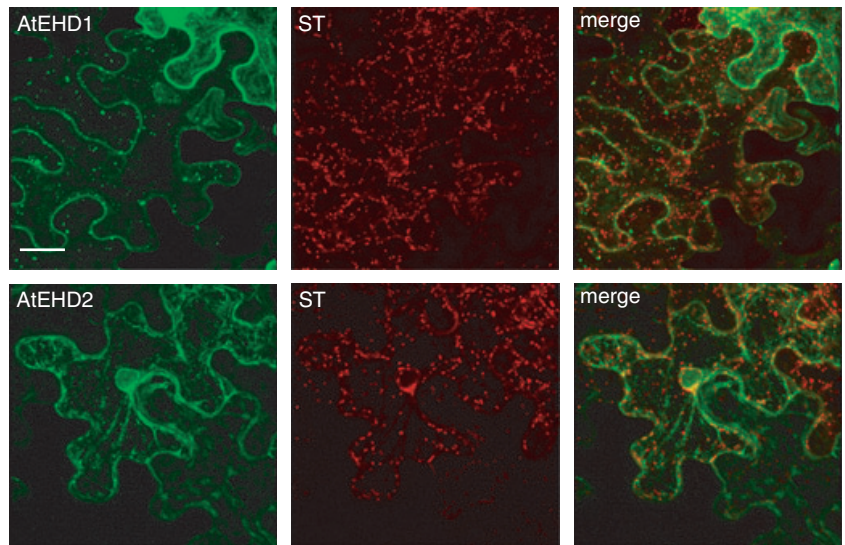


Figure 4. Intracellular localization of Arabidopsis Eps15 homology (EH) domain-containing protein (AtEHD1) and the Golgi marker.

Tobacco leaves were infiltrated with a mixture of *Agrobacterium tumefaciens* ($\text{OD}_{600} = 0.1$) containing Pro_{35S}:AtEHD1-GFP (upper panel) and Pro_{35S}:ST-mRFP (Golgi marker) or Pro_{35S}:AtEHD2-GFP and Pro_{35S}:ST-mRFP. Leaf sections were visualized 48 h after injection under a laser-scanning confocal microscope (Zeiss). Scale bar represents 10 μm .



and Drobak, 2002; Jensen *et al.*, 2001; Voigt *et al.*, 2005; Golomb *et al.*, 2008). Ara-6, a small GTPase unique to plants (Ueda *et al.*, 2001), was found to be localized to the early endosome in plant cells. The FYVE domain has been reported to localize to endosomes in mammals (Stenmark *et al.*, 1996) as well as plants (Heras and Drobak, 2002; Jensen *et al.*, 2001; Voigt *et al.*, 2005; Golomb *et al.*, 2008). The co-localization of AtEHD1-yellow fluorescent protein (YFP) with Ara6-cyan fluorescent protein (CFP) and AtEHD1-GFP with FYVE-DsRed (Voigt *et al.*, 2005) was examined by transient expression assays and is shown in Figure 5. As can be seen, a sub-population of the dot-like structures which were positive for Ara6 or FYVE were also positive for AtEHD1, confirming the localization of AtEHD1 on endosomes. In the case of both FYVE and ARA6, the endosomes on which AtEHD1 co-localized with these markers were

usually in the vicinity of the plasma membrane and were not usually observed in the upper 'endosomal' focal plane as in Figure 2(a) for example. Clearly, the co-localization of AtEHD1 with these markers occurs in a sub-population of endosomes. It seems that a sub-population of FYVE- and/or ARA6-positive endosomes are AtEHD1 positive; likewise, not all AtEHD1 endosomes are positive for either marker. It is therefore possible that not all AtEHD1-positive endosomes have the same function or composition. The expression levels of the different proteins could also be affecting the extent of the co-localization. This needs to be further investigated. However, as demonstrated in Figure S4, when AtEHD1-GFP pixel intensities and ARA6-CFP or FYVE-DsRed intensities of individual vesicular structures were aligned against each other it was evident that AtEHD1 is co-localized with the two endosomal markers.

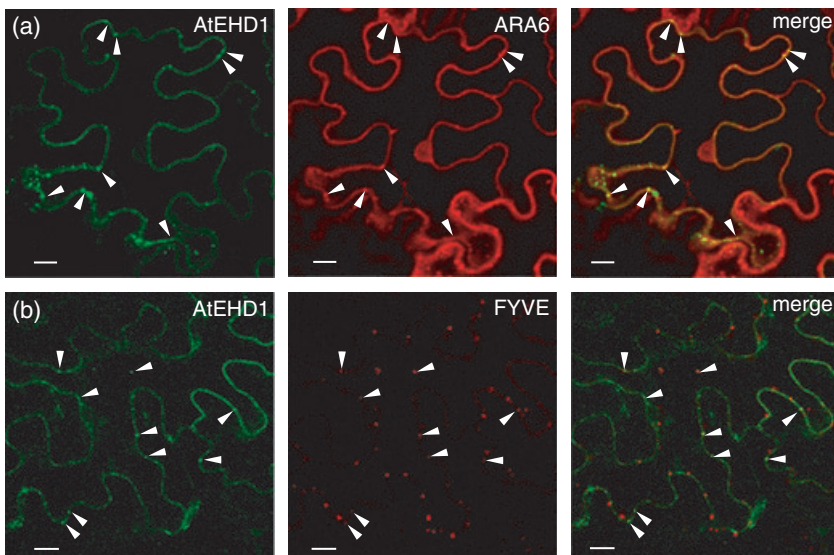


Figure 5. Co-localization of Arabidopsis Eps15 homology (EH) domain-containing protein (AtEHD1) with endosome markers Ara6 and FYVE. Tobacco leaves were infiltrated with a mixture of *Agrobacterium tumefaciens* ($OD_{600} = 0.1$) containing Pro_{35S}:AtEHD1-YFP and Pro_{35S}:Ara6-CFP (upper panel) or Pro_{35S}:AtEHD1-GFP and FYVE-DsRed (lower panel). Leaf sections were visualized 48 h after injection under a laser-scanning confocal microscope (Zeiss). Scale bar represents 10 μ m. Triangles indicate co-localized pixels.

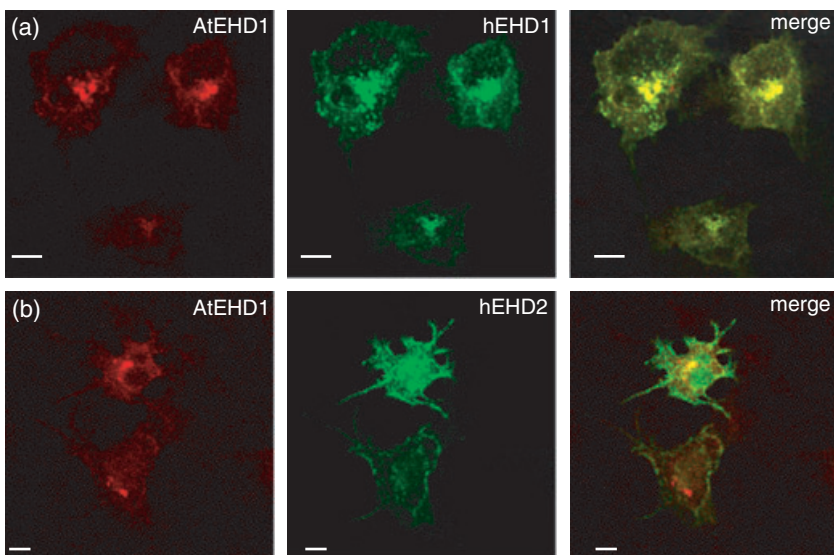


Figure 6. Intracellular localization of Arabidopsis Eps15 homology (EH) domain-containing protein (AtEHD1) in mammalian cells. COS-7 cells were (a) co-transfected with AtEHD1 and hEHD1; (b) co-transfected with AtEHD1 and hEHD2, using the JetPEI® reagent. Sixteen hours post-transfection cells were fixed with 4% paraformaldehyde and visualized under a laser-scanning confocal microscope (Zeiss). Scale bar represents 20 μ m.

Localization of AtEHD proteins in mammalian cells

As a first step in testing the possible functional similarity between Arabidopsis EHD1 and mammalian EHDs we performed localization assays of AtEHD1 in mammalian cells (Figure 6). COS-7 cells were transfected with *AtEHD1-mRFP* (Figure S5) or co-transfected with *AtEHD1-mRFP* and *hEHD1-GFP* (Figure 6a) or with *AtEHD1-mRFP* and *hEHD2-GFP* (Figure 6b). AtEHD1 localized primarily to a distinct peri-nuclear structure, which was also positive for transferrin staining (not shown), indicating it is the ERC. As is evident from Figure 6(a), AtEHD1 appears to be co-localized with hEHD1, while it does not co-localize with hEHD2 (Figure 6b). The location of the GFP or mRFP in the chimeric proteins (C-terminal or N-terminal) did not affect the localization of the chimeric protein (data not shown). Additionally, the

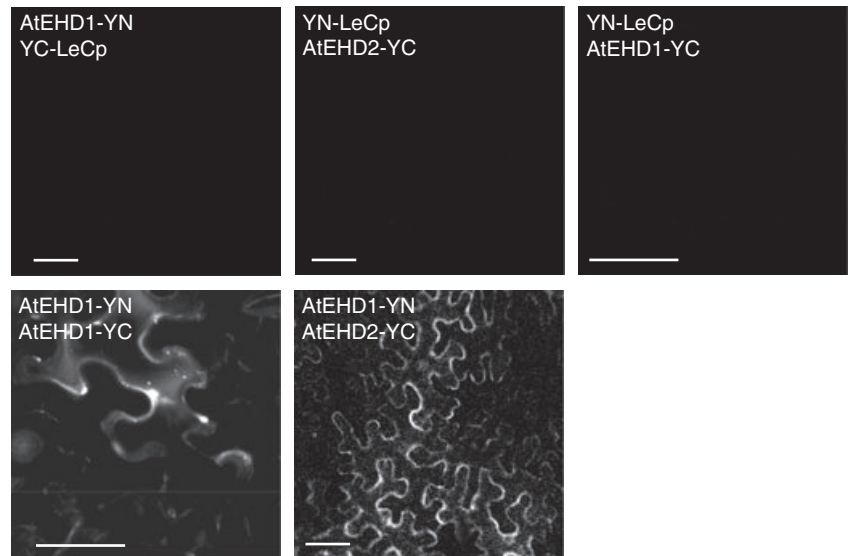
results achieved in BHK and HELA cells were similar to those achieved with COS-7 cells (data not shown). The distinct peri-nuclear localization observed in mammalian cells for human EHD1 and EHD3 is known to be localized to the ERC (Mintz *et al.*, 1999). Mammalian EHD1 and EHD3 are localized to the ERC and EHD1 controls recycling from the ERC to the plasma membrane (Rapaport *et al.*, 2006). Figure S6 presents the full localization panels for both AtEHD proteins with all four mammalian EHDs. As observed, AtEHD1 co-localizes with both hEHD1 and hEHD3, while AtEHD2 does not fully co-localize with any of the mammalian EHDs.

Interaction between AtEHDs

Mammalian EHDs are known to associate with each other and undergo hetero- and homo-dimerization (Galperin

Figure 7. Bimolecular fluorescence complementation (BiFC) visualization of the interaction between Arabidopsis Eps15 homology (EH) domain-containing protein 1 (AtEHD1) and AtEHD2.

Fluorescence images of *Nicotiana benthamiana* leaves infiltrated with a mixture of *Agrobacterium tumefaciens* ($OD_{600} = 0.1$) containing Pro_{35S}:YN-AtEHD1, Pro_{35S}:YC-AtEHD2, Pro_{35S}:YN-LeCp (Matarasso *et al.*, 2005) and Pro_{35S}:YC-LeCp as indicated. Leaf sections were visualized 48 h after injection under a laser-scanning confocal microscope (Zeiss). Scale bar represents 50 μm .



et al., 2002; Lee *et al.*, 2005). Interactions between AtEHD1 and AtEHD2 *in planta* were examined directly by bimolecular fluorescence complementation (BiFC) analysis, in which active YFP is reconstituted only when non-fluorescent N-terminal (YN) and C-terminal (YC) YFP fragments are brought together by protein–protein (AtEHD1–AtEHD2) interactions (Bracha-Drori *et al.*, 2004). Reconstitution of YFP fluorescence was examined by transient co-expression of the tested protein pairs. Plants were transformed by infiltration of *Agrobacterium tumefaciens* harboring the appropriate plasmid to the abaxial side of *Nicotiana benthamiana* leaves. Cells co-expressing YN-AtEHD1 and YC-AtEHD2 showed clear YFP fluorescence (Figure 7). Interestingly, AtEHD1 also homodimerizes, and the interaction is localized to the PM as well as on vesicles (Figure 7). AtEHD2 appears to also homodimerize but the fluorescence was weak, possibly due to expression levels or the interaction being weak (not shown). Notably, it has recently been published that hEHD4 homodimerizes and heterodimerizes with hEHD2 weakly as compared with its heterodimerization with hEHD1 or hEHD3 (Sharma *et al.*, 2008). YN-AtEHD1, YN-AtEHD2, YC-AtEHD1 and YC-AtEHD2 were examined for fluorescence with the complementary half of the YFP protein and the results were negative. Additionally, all fusion proteins employed in the assay were examined for interaction with a Solanaceae cysteine protease (Matarasso *et al.*, 2005) which is similar in size to the AtEHDs as well as the Arabidopsis diminuto/DWARF1 (Klahre *et al.*, 1998) protein and the results were negative, indicating that the interaction is specific.

We applied the yeast two-hybrid system to confirm the results obtained *in planta*. In the yeast system we found that AtEHD1 interacts with AtEHD2. Moreover, each of the AtEHDs can also homodimerize (Figure S7), similarly to the mammalian proteins, though the homodimerization of

AtEHD2 is weak, consistent with the BiFC results. Yeast strains carrying AtEHD1 or AtEHD2 in the bait and AtEHD1 or AtEHD2 in the prey grew in the absence of leucine, indicating LEU2 reporter gene activation. When grown on X-gal plates, these yeast cells were blue as a result of activation of the LacZ reporter gene. In contrast, a control yeast strain expressing the arbitrary bait, Bicoid (LexA fused to a transcriptionally inert fragment of the *Drosophila* Bicoid product), and the AtEHD prey did not activate the LEU2 or LacZ reporter genes (Figure S7). Expression was dependent upon growth on galactose medium, indicating that expression of AtEHDs in the prey was required for expression of the reporter genes.

Functional phenotypes of silenced AtEHD1/AtEHD2 Arabidopsis plants

In order to further study the role of AtEHD proteins in plants, we used over-expression and silencing approaches. We attempted to generate transgenic Arabidopsis plants over-expressing either AtEHD1 or AtEHD2, respectively, under the control of the 35S promoter. Despite several attempts, it was not possible to obtain Arabidopsis plants which over-expressed AtEHD2. The transgenic Arabidopsis plants over-expressing AtEHD1 did not show a detectably different phenotype from wild-type control plants (data not shown). Though knock-out T-DNA insertion lines reportedly exist at SALK, the seeds were obtained but turned out to be wild-type seeds or heterozygous seeds from which homozygote knock-out plants could not be obtained despite several attempts. We applied the RNAi approach to silence *AtEHD* expression. Several transgenic lines of each silenced gene were examined for silencing with semi-quantitative RT-PCR (Figure 8a) as well as phenotypically. The silenced plants were viable and fertile, though they tended to flower early in

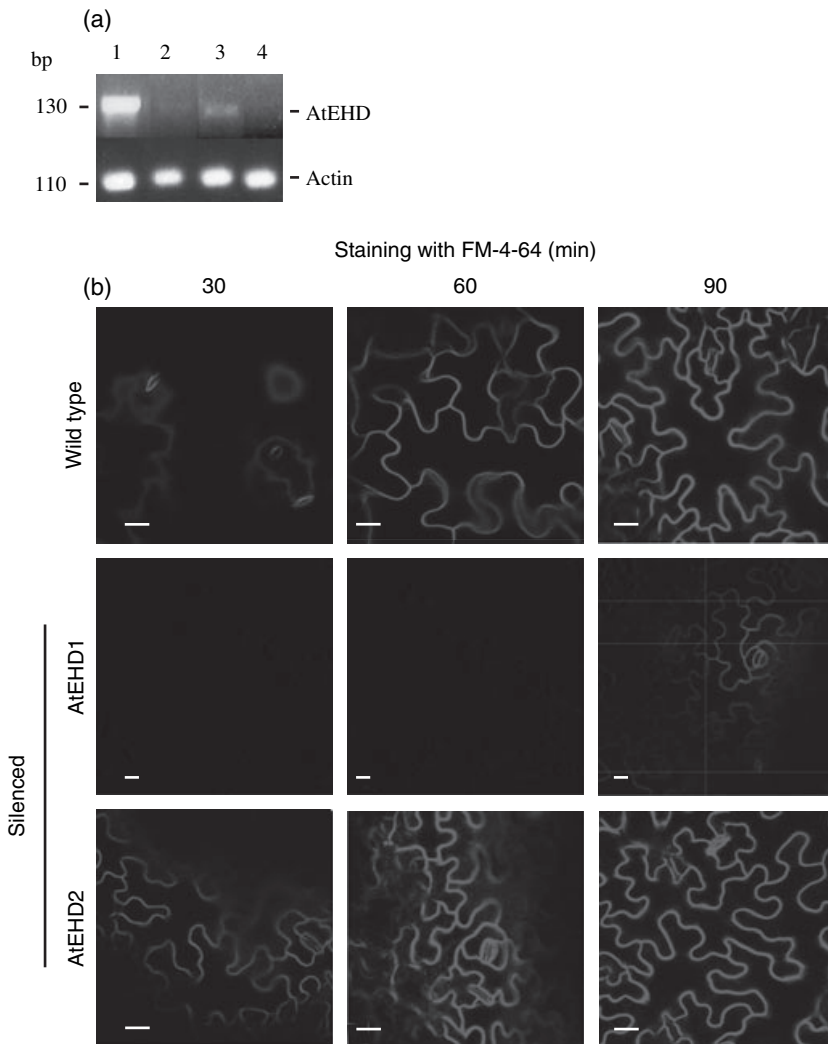


Figure 8. Internalization of FM-4-64 in Arabidopsis Eps15 homology (EH) domain-containing protein (AtEHD) silenced Arabidopsis plants.

(a) Total RNA was isolated from control and silenced Arabidopsis plants and the expression of AtEHD1 and AtEHD2 was analysed by RT-PCR. Ethidium bromide-stained 1% agarose gel showing RT-PCR products (30 cycles) using: (lane 1) wild type and (lane 2) AtEHD1 silenced plants with *AtEHD1*-specific primers, (lane 3) wild type and (lane 4) AtEHD2 silenced plants with *AtEHD2*-specific primers. Actin-specific primers were used as control in all cases.

(b) Five-week-old silenced Arabidopsis plants as indicated were injected with $10 \mu\text{M}$ FM-4-64. Leaf sections were visualized post-injection under a laser-scanning confocal microscope (Zeiss). Scale bar represents $10 \mu\text{m}$.

short-day growth conditions, especially the strongly silenced lines. This could indicate that silencing of either AtEHD causes stress, or could possibly relate to the known connection between endocytosis and plant hormone shuttling and metabolism (Paciorek *et al.*, 2005). There was no particular phenotype observed in the silenced lines, except for the tendency towards early flowering. Several lines of the two silenced plants showed similar growth. The sizes of the plants were indistinguishable. They showed similar leaf size and similar numbers of leaves. This was also the case upon comparison with wild-type plants, except for the aforementioned early-flowering effect.

Silencing the expression of *AtEHD1* in Arabidopsis plants caused delay in the plasma membrane labeling of the fluorescent dye FM-4-64. This could indicate slower internalization or possibly a delay in other processes which affect the entrance or labeling of the dye (Figure 8; all sections were captured with identical gain and offset). Silencing of *AtEHD2* (and most likely *AtEHD2-2* as well) did not affect the

labeling of the plasma membrane with FM-4-64 (Figure 8b). *mEHD1* knock-out mice showed moderately delayed recycling (Rapaport *et al.*, 2006), which may be similar to that observed in the silenced *AtEHD1* Arabidopsis plants. The delay in recycling/internalization in both cases is apparently not severe enough to impede development or cause any other noticeable negative phenotype. The fact that the silenced plants are normal, similarly to *mEHD1* knock-out mice (Rapaport *et al.*, 2006) indicates that the two proteins, or rather the two loci, can sufficiently complement each other to maintain the required cellular functions. Alternatively, additional unidentified plant proteins may compensate for the absence of AtEHD1.

Suppressive effect of AtEHD2 on endocytosis

The FM-4-64 staining assay and a transferrin internalization assay were employed in order to examine the effect of plant EHD proteins on endocytosis. Interestingly, transferrin

internalization was recently shown to occur in Arabidopsis protoplasts expressing the human transferrin receptor (Ortiz-Zapater *et al.*, 2006).

Nicotiana tabacum cv. Samsun cell cultures (in the exponential growth phase) expressing AtEHD2-GFP were incubated for different times with FM-4-64 at a final concentration of 10 μM . Cells were examined under a laser-scanning confocal microscope at desired time points for GFP and FM-4-64 fluorescence. High expression of AtEHD2 inhibits the internalization of the dye (Figure 9a).

Similarly, we tested the effect of over-expressing AtEHD2 in mammalian cells on transferrin internalization. Fourteen hours after *AtEHD2-GFP* transfection of COS-7 cells, endocytosis of Texas-Red-conjugated transferrin was examined. Transferrin (4 $\mu\text{g ml}^{-1}$) was added for times ranging from 2 to 20 min and cells were then acid washed to remove any bound but not internalized material from the surface of the cells. The cells were fixed and visualized using confocal microscopy. The results (Figure 9b) indicate that AtEHD2 has an inhibitory effect on endocytosis when over-expressed, similarly to over-expression of hEHD2 (Guilherme *et al.*, 2004).

Quantitative results from at least 200 cells for each measurement are depicted in Figure 9. Evidently AtEHD2 has an inhibitory effect on endocytosis when over-expressed, in both plant and mammalian cells. Representative cell pictures are included. In Figure 9(a), the 'low' expressing row of cells at the bottom of the panel internalized more FM-4-64 than the two upper rows of cells which express AtEHD2 more strongly. In Figure 9(b), the general field of cells which do not express AtEHD2 and the cells numbered '1' and '2' which have a low level of AtEHD2 expression internalized more transferrin than the highly expressing cells (outlined in the middle panel). AtEHD1 was also examined in both systems but did not significantly affect the rate of internalization of FM-4-64 in plant cells or of transferrin in mammalian cells (not shown).

Discussion

The present work describes the isolation of plant proteins homologous to the mammalian EHD proteins, and demonstrates a role for said proteins in endocytosis. Sequence analysis and preliminary functional characterization of AtEHDs suggests a high level of functional homology with mammalian EHDs. Analysis of the databases and RT-PCR conducted on total cDNA demonstrated the existence of EHD orthologs in Arabidopsis and the entire Solanaceae family as well as in rice, maize and basically all plant species examined. The Arabidopsis EHD proteins share high homology with their mammalian counterparts (Galperin *et al.*, 2002; Mintz *et al.*, 1999; Pohl *et al.*, 2000). Structurally, AtEHD1, AtEHD2 and AtEHD2-2 contain the same domains as the mammalian proteins (Galperin *et al.*, 2002;

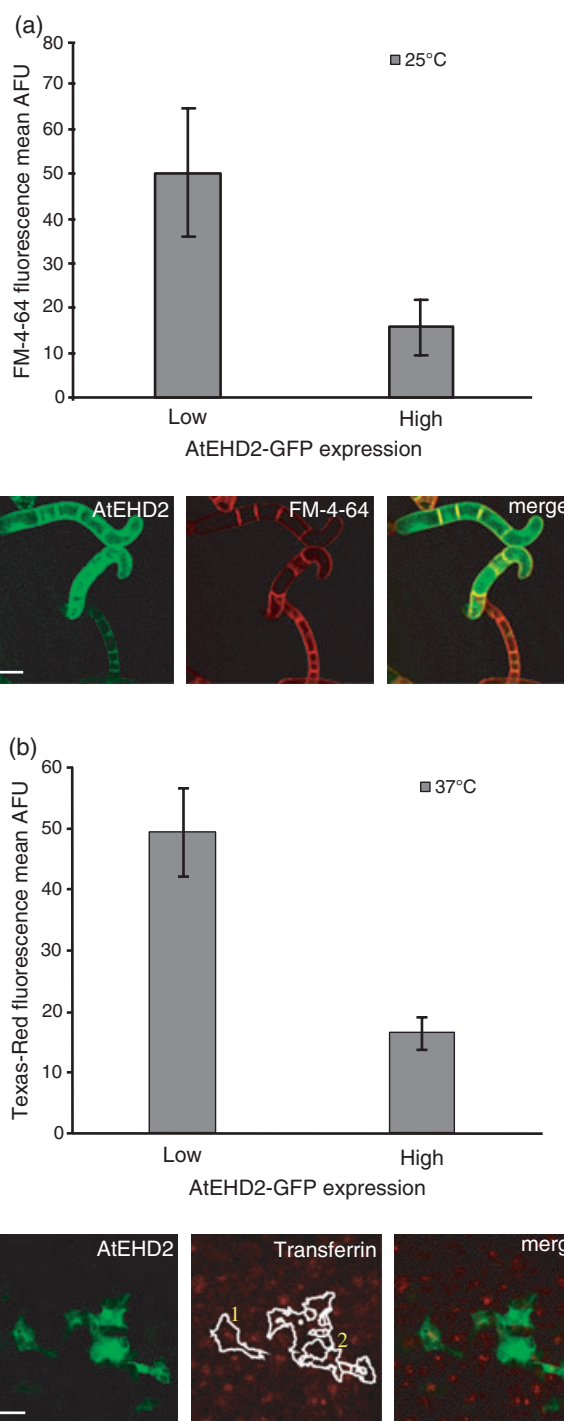


Figure 9. Inhibitory effect of over-expression of Arabidopsis Eps15 homology (EH) domain-containing protein 2 (AtEHD2) on internalization. (a) FM 4-64 internalization in a tobacco culture expressing AtEHD2-GFP. (b) Transferrin internalization in COS-7 cells expressing AtEHD2. Cells were visualized under a laser-scanning confocal microscope (Zeiss). The scale bar in both cases represents 20 μm . Graphs were generated from over 200 cells for each point, using ImageJ software. 'Low' expression = less than 15 GFP arbitrary fluorescent units (AFU); 'High' expression = over 15 GFP AFU. Representative sections of microscopy data used for the analysis are shown in both (a) and (b). In (b), cells showing high expression and low expression (1, 2) of AtEHD2 are outlined in the middle panel.

Naslavsky and Caplan, 2005; Pohl *et al.*, 2000). The EHD is present at the N-terminus of the plant proteins, with the central domain harboring the nucleotide-binding site and the DxxG motif that is completely conserved in evolution (Galperin *et al.*, 2002; Rotem-Yehudar *et al.*, 2001). However, the conserved NKxD motif is missing in the plant EHD proteins. The second difference between the plant EHD proteins and the mammalian, *Drosophila* and *C. elegans* proteins, is the location of the EH. While many N-terminal EHD-containing proteins exist in mammals and other organisms (Naslavsky and Caplan, 2005), the AtEHs described herein bear the most resemblance (structurally and in terms of sequence) to the EHDs. Additional EHD-containing proteins, which are more similar to the N-terminal EHD-containing proteins of mammals exist in plants (for example, there are 13 accessions of EHD-containing proteins in rice, as well as an Arabidopsis Eps15 homolog) and are undoubtedly involved in aspects of the endocytic process as well. The conservation of EHD proteins throughout the plant kingdom and their relatively high homology to the mammalian counterparts provides a clue to their relative importance in plant systems. Despite the difference in domain arrangement, we demonstrate herein that it is highly likely that the plant EHD proteins have a similar function to their mammalian counterparts. Interestingly, available microarray data (Zimmermann *et al.*, 2004) indicate that the Arabidopsis EHD genes are expressed in all plant tissues. This correlates with the data obtained for the mammalian EHD proteins (George *et al.*, 2007; Mintz *et al.*, 1999; Pohl *et al.*, 2000; Rapaport *et al.*, 2006), and may also indicate the importance of EHDs in functions which occur in all types of cells. Given the role of EHDs in endocytosis, it is likely that these conserved proteins serve integral roles in signaling in a variety of cell types among diverse species (Polo *et al.*, 2003). Notably, the mammalian EHD2 protein appears to be the EHD protein most unique to mammals, and the same is true for AtEHD2 in Arabidopsis. This is interesting, given that, in both cases, EHD2 is not endosomal and was the only EHD found to inhibit endocytosis in mammals as well as in Arabidopsis as demonstrated herein.

As shown above, in Arabidopsis, two separate loci encode at least three EHD proteins. We showed that two of the plant EHDs are localized to endocytic organelles and co-localize with endocytic markers in both plant and mammalian systems. The fact that both proteins co-localize with FM-4-64 shows that they are localized to membranal endocytic organelles; this was further confirmed by the fact that silenced AtEHD1 plants are delayed in FM-4-64 internalization. Co-localization of AtEHD1 with ARA6 and FYVE seems to suggest that AtEHD1 may localize to early endosomes, though better characterization of plant endocytic compartments and endocytosis markers is needed in order to make such a determination.

AtEHD1 fully co-localizes with hEHD1 and hEHD3 but does not co-localize with hEHD2 or hEHD4. This could indicate that AtEHD1 and hEHD1/3 share similar functions, and is consistent with reported phenotypes for hEHD1 knock-out mice. Interestingly, AtEHD2 does not significantly co-localize with any of the mammalian EHDs. Though plant proteins are by no means guaranteed to localize properly in mammalian cells, it would seem that AtEHD2 shares similar functions with hEHD2 based on the inhibitory effect on endocytosis, an effect which was also observed with AtEHD2 in a mammalian system using mammalian cells, indicating that AtEHD2 is able to exert at least some of its native biological activity in mammalian cells. To put it another way, AtEHD2 is able to exert similar biological activities in both plant and mammalian cells, a fact which testifies to the high level of functional homology between plant and mammalian endocytosis, with such homology being increasingly confirmed as of late (Lam *et al.*, 2007; Ortiz-Zapater *et al.*, 2006; Dhonukshe *et al.*, 2007).

Silenced AtEHD1 Arabidopsis plants demonstrated a delay in internalization of the fluorescent dye FM-4-64. As AtEHD1 co-localizes with hEHD1, it is possible that this delay may be a similar phenomenon to the delayed recycling observed in mhEHD1 knock-out mice (Rapaport *et al.*, 2006). Silenced AtEHD1 plants did not show any distinctive phenotype, similarly to knock-out mice (Rapaport *et al.*, 2006). It is possible that unidentified plant proteins compensate for the loss of AtEHD1 and therefore there is no distinctive phenotype in silenced AtEHD1 plants. By contrast, AtEHD2 over-expression suppresses endocytosis in both plant and mammalian cells, as does hEHD2 in mammalian cells (Guilherme *et al.*, 2004). It is thus possible that AtEHD1 and AtEHD2 have co-evolved in plants to exert opposite effects; one may act to stimulate endocytosis under certain conditions, while the other can suppress endocytosis under certain conditions. The rate of endocytosis or the amount of material allowed to enter the cell are parameters which depend on a multitude of factors, many of which remain unknown. However, these parameters could be influenced by the level of expression (or other regulatory elements) of one or both AtEHs. One could envisage a decrease of active AtEHD1 or an increase of active AtEHD2 in a situation where endocytosis must be limited, or vice versa. Much additional work is needed in order to examine this possibility.

Further, in connection with the inhibitory effect of AtEHD2 over-expression on endocytosis it is possible that AtEHD2 is involved in a particular rate-limiting step of the endocytic process. In such a case, over-expression of AtEHD2 may cause the endocytic process to become 'stuck' in this particular step throughout the cell, and thus inhibit the normally faster entry of typically endocytosed material into the cell. This could also explain why the expression level of *AtEHD2* is normally low in wild-type cells compared with the expression of *AtEHD1*.

In conclusion, localization, co-localization and functional analysis of plant EHD proteins strongly indicate that they are involved in endocytosis in plants. While the exact mediators and localities of clathrin-dependent endocytosis in plants have yet to be elucidated, the fact that AtEHD1 localizes to mammalian endocytic organelles and AtEHD2 influences transferrin internalization in itself testifies to the high level of functional homology between the mammalian and plant endocytic pathways. The exact function of each plant EHD protein within the endocytic process remains to be solved.

Experimental procedures

Plant and cell culture material and growth conditions

Nicotiana tabacum cv Samsun and *A. thaliana* cv Columbia were grown from seeds under greenhouse conditions. *Nicotiana tabacum* cv. Samsun cells were maintained by weekly dilution in fresh MS medium (Murashige and Skoog, 1962) supplemented with $100 \mu\text{g L}^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D) and 30 g L^{-1} sucrose. The cells were maintained with shaking at 110 rev min^{-1} at 25°C .

Transgenic plants were first germinated on the appropriate sterile selective solid medium and transferred to soil 2–4 weeks after germination.

Construction of expression plasmids

AtEHD1 and *AtEHD2* cDNAs were cloned independently in the sense orientation upstream of the *GFP* gene into the binary vector pBIN-PLUS between the 35S- Ω promoter containing the translation enhancer signal and the Nos terminator generating *Pro*_{35S}:*AtEHD1-GFP* and *Pro*_{35S}:*AtEHD2-GFP* constructs, respectively. The constructs were electroporated into *A. tumefaciens* GV3101 and the bacteria used for transient expression assays.

To visualize the expression of AtEHD proteins in mammalian cells, *AtEHD1* and *AtEHD2* were cloned in the sense orientation into the mammalian expression vector pEGFP-N1 (Clontech, <http://www.clontech.com/>). Constructs encoding chimeric fluorescent fusion proteins were tested in COS-7 and/or BHK cells.

For silencing in Arabidopsis, a segment of *AtEHD1* (474 bp from residue 1 to residue 474) and *AtEHD2* (447 bp from residue 1 to residue 447) were separately cloned in the pKANNIBAL vector in both the sense and the anti-sense orientation, flanking the Pdk intron (Wesley *et al.*, 2001). These constructs were sub-cloned into the binary vector pART27 (Gleave, 1992) and used for transforming Arabidopsis plants. The region chosen differs significantly between the two genes, and does not contain a stretch of homologous nucleotides large enough to be processed into siRNA for either gene.

Stable and transient transformation

Nicotiana tabacum cv. Samsun plants were transformed as previously described (Horsch *et al.*, 1985). Arabidopsis plants were transformed as previously described (Clough and Bent, 1998).

Transient expression was performed as previously described (Ron and Avni, 2004). Briefly, the *AtEHD* constructs were cloned in pBINplus (van Engelen *et al.*, 1995) and introduced by electroporation into *A. tumefaciens* strain GV3101. *Agrobacterium* were grown in Luria–Bertani (LB) medium overnight, diluted into an induction

medium [50 mM MES pH 5.6, 0.5% (w/v) glucose, 1.7 mM NaH_2PO_4 , 20 mM NH_4Cl , 1.2 mM MgSO_4 , 2 mM KCl, $17 \mu\text{M}$ FeSO_4 , $70 \mu\text{M}$ CaCl_2 and $200 \mu\text{M}$ acetosyringone] and grown for an additional 6 h until the OD_{600} reached 0.4–0.5. The *Agrobacterium* culture was diluted to $\text{OD}_{600} = 0.05$ – 0.2 , and the suspensions were injected with a needleless syringe into the leaves of 7- to 8-week-old tobacco plants. Leaves were observed for protein expression 24–72 h after injection.

Generation of cell cultures from transgenic plants was carried as described by Ron and Avni (2004).

Mammalian cell culture growth conditions and transfection

COS-7 cells expressing the SV40 large T antigen were grown on Dulbecco's modified Eagle's medium (DMEM) + L-Glu (Invitrogen, <http://www.invitrogen.com/>) supplemented with 10% Fetal Bovine Serum (Invitrogen) and 1% Pen/Strep (Invitrogen), at 37°C in a 5% CO_2 incubator and sub-cultured 1:8–1:12 twice weekly.

Transfections of COS-7 cells were carried out using JetPei[®] according to the JetPei[®] user manual (Poly-Plus transfection, <http://www.polyplus-transfection.com/>).

Confocal microscopy

Cells were analyzed using a 510 Zeiss confocal laser scanning microscope (Zeiss, <http://www.zeiss.com/>) with the following configuration: 30 mW argon and HeNe lasers, 488 and 568 maximum lines. All images depict single sections. Contrast and intensity for each image were manipulated uniformly using Adobe Photoshop and/or ImageJ software.

Cellular fractionation

Nicotiana tabacum cv. Samsun plants over-expressing *AtEHD1* or *AtEHD2* were ground in liquid nitrogen. Cell fractionation was carried out as previously described (Bar-Peled and Raikhel, 1997). Briefly, 2 g of tobacco leaves expressing *AtEHD1* or *AtEHD2* were ground on ice with a mortar and pestle in 4 ml cold lysis buffer (100 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS)-HCl, pH 7.5, 0.4 M sucrose, 10 mM potassium acetate and 1 mM EDTA), 8 μl of 1 M DTT and 20 μl of 0.1 M polymethylsulfanyl fluoride (PMSF). Samples were placed on a shaker (100 rpm) for up to 15 min at 4°C . The homogenate was termed total crude homogenate.

Total crude homogenate was centrifuged for 60 min at 150 000 g, generating a total membrane pellet (termed P150) and a soluble protein fraction (termed S150). Pellets (P150) were rinsed briefly with 1 ml of lysis buffer and further resuspended in lysis buffer containing 150 mM NaCl or 1% Triton X-100, or 1% sarcosyl, or 0.1 M Na_2CO_3 . After 30 min on ice, the solubilized pellets (P150) were spun (at 150 000 g for 15 min) to remove undissolved matter, and the supernatant was saved for analysis.

Endocytosis of transferrin and FM-4-64

Staining of tobacco cells with FM-4-64 was performed as previously described (Bloch *et al.*, 2005; Bolte *et al.*, 2004). Briefly, FM-4-64 at a concentration of $5 \mu\text{M}$ was injected into the abaxial side of *N. benthamiana* or *A. thaliana* using a needleless syringe. Cells were examined under a laser-scanning confocal microscope at the desired time points for FM-4-64 staining. Roots of 1- to 2-week-old Arabidopsis seedlings were submerged in a solution of $5 \mu\text{M}$ FM-4-64 for the desired time points. FM 4-64 staining was examined under

a confocal laser scanning microscope. *Nicotiana tabacum* cv. Samsun cell cultures were incubated with 10 μM FM-4-64 for the indicated time points. FM 4-64 staining was examined under a confocal laser scanning microscope.

Transfected COS-7 cells, grown on cover slips, were incubated for 30 min in binding medium (DMEM, 0.1% BSA, 20 mM HEPES, pH 7.2) to deplete the transferrin present in the medium. Following incubation with 4 $\mu\text{g ml}^{-1}$ (final) Texas-Red-conjugated transferrin (Invitrogen), cells were rapidly cooled to 4°C, acid washed, washed with cold phosphate buffered saline (PBS) and fixed with 3.7% paraformaldehyde. The fixed cells were mounted for microscopy.

Bimolecular fluorescence complementation analysis

AtEHD1 and *AtEHD2* cDNA fragments were sub-cloned into the *Spel* sites of pSY751 containing the N-terminal fragment of the YFP (YN), and pSY752 vectors contain the C-terminal fragment of the YFP (YC) (Bracha-Drori *et al.*, 2004). The resulting plasmids, pSY751-*AtEHD1* (YN-*AtEHD1*) and pSY752-*AtEHD2* (YC-*AtEHD2*), were used for transient expression assays in *N. benthamiana* leaves. After incubation at 24°C for 48 h, the epidermal cell layers were viewed under a confocal microscope.

RT-PCR analysis of Arabidopsis *AtEHD1* and *AtEHD2* expression

Total RNA was extracted from leaves of 3- to 4-week-old Arabidopsis plants. Five micrograms of RNA was converted to cDNA using M-MLV reverse transcriptase (Promega, <http://www.promega.com/>). Two microliters of each reverse transcriptase reaction were used as a template in a 50- μl PCR reaction containing specific primers. A 753-bp fragment of *AtEHD1* was amplified using the following primers: ATG GAG ATC GAA TCC GTC GC (forward) and CTG CTT GAA CTG CTA CTG TG (reverse); a 743-bp fragment of *AtEHD2* was amplified using the following primers: ATG GAG ACT TCA TCG ACG (forward) and GCA ACT ACA AAC CTA TCT GTT GTT G (reverse). Actin-specific primers were used as a control.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Figure S1. Stable Arabidopsis transgenic plants expressing Arabidopsis Eps15 homology (EH) domain-containing protein 1 (*AtEHD1*)-GFP.

Figure S2. Localization of the endocytic marker FM-4-64 in leaf tissue.

Figure S3. Localization of the endocytic marker FM-4-64 in root tissue.

Figure S4. Co-localization of Arabidopsis Eps15 homology (EH) domain-containing protein 1 (*AtEHD1*) with endosome markers Ara6 and FYVE.

Figure S5. Intracellular localization of Arabidopsis Eps15 homology (EH) domain-containing protein 1 (*AtEHD1*) in mammalian cells.

Figure S6. Intracellular localization of Arabidopsis Eps15 homology (EH) domain-containing protein 1 (*AtEHD1*) and *AtEHD2* in mammalian cells.

Figure S7. Interaction between Arabidopsis Eps15 homology (EH) domain-containing protein 1 (*AtEHD1*) and *AtEHD2* in a yeast two-hybrid system.

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