

# A Novel Localization Pattern for an EB1-like Protein Links Microtubule Dynamics to Endomembrane Organization

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## Summary

A group of microtubule-associated proteins called +TIPs (plus end tracking proteins), including EB1 family proteins [1], label growing microtubule ends specifically in diverse organisms [2, 3] and are implicated in spindle dynamics [4], chromosome segregation [5], and directing microtubules toward cortical sites [6]. Here, we report three new EB1-like proteins from *Arabidopsis* and provide the intracellular localization for AtEB1, which differs from all known EB1 proteins in having a very long acidic C-terminal tail. In marked contrast to other EB1 proteins, the GFP-AtEB1 fusion protein localizes not only to microtubule plus ends but also to motile, pleiomorphic tubulovesicular membrane networks that surround other organelles and frequently merge with the endoplasmic reticulum. AtEB1 behavior thus resembles that of +TIPs, such as the cytoplasmic linker protein CLIP-170, that are known to associate with and pull along membrane tubules in animal systems [7, 8] but for which homologs have not been identified in plants. In addition, though EB1 proteins are believed to stabilize microtubules [3, 9], a different behavior is observed for AtEB1 where instead of stabilizing a microtubule it localizes to already stabilized regions on a microtubule. The dual localization pattern of AtEB1 suggests links between microtubule plus end dynamics and endomembrane organization during polarized growth of plant cells.

## Results and Discussion

A BLASTP search of the *Arabidopsis* database using the mouse adenomatous polyposis coli (APC) binding EB1 (end binding 1) gene (gi: 1256434) revealed three EB1-like genes (At5g62500, At5g67270, and At3g47690; Figure 1). These genes, designated as AtEB1 (At5g62500), AtEB1-Homolog1 (At5g67270), and AtEB1-Homolog2 (At3g47690) transcribing cDNAs of 882 bp, 990 bp, and 468 bp, respectively, display up to 67% amino acid similarity among themselves and between 39% to 59% similarity with EB1 orthologs from other organisms (Figure 1). The *Arabidopsis* EB1-like proteins share a common

N-terminal type-2 calponin homology (CH) domain [10, 11] with other homologs. This domain is the first example of a single CH domain that can associate with microtubules [12]. Certain leucine residues are also conserved between the homologs (asterisks, Figure 1) suggesting a basic domain-leucine zipper motif (bZIP) similar to that of transcriptional activators, such as Maf, Fos, Jun, and Myc families [13, 14]. The C-terminal regions of the different EB1 proteins are more divergent (Figure 1) with AtEB1 showing an especially long stretch of acidic (glutamic acid) residues (Figure 1). The presence of a highly polar acidic tail suggests that AtEB1 may be involved in forming a complex with basic microtubule-associated proteins (MAPs) and indeed for human EB1 the residues 134–268 toward the C terminus have been shown to constitute the APC binding region [15]. Alternatively, tubulin modification through the addition of one to five glutamic acid residues to the C-terminal variable region of some  $\alpha$ - and  $\beta$ -tubulins results in a negatively charged “bottle brush” structure protruding from the microtubule surface [16]. The role of such a structure is unclear. AtEB1 was therefore developed as a GFP-fusion probe to visualize and understand its in vivo behavior.

## GFP-AtEB1 Exhibits Dual Localization to Microtubules and Membranes

An inframe N-terminal fusion created between a green fluorescent protein (GFP) and the 882 bp AtEB1-cDNA was placed under the control of a CaMV35S promoter and introduced into *Arabidopsis* plants (ecotype Wassilewskija). So far all the localization patterns described for EB1 family members have established them as microtubule plus end binding proteins. However, overexpression of EB1-proteins labels microtubules extensively and is reported to increase their stability [3, 17]. We were therefore concerned whether expression of GFP-AtEB1 under the strong CaMV35S promoter would stabilize microtubules in transgenic *Arabidopsis* plants. However, increased microtubule stability/bundling produces a specific swollen epidermal cell phenotype in *Arabidopsis* [18]. Transgenic *Arabidopsis* lines carrying the fusion protein were therefore prescreened for normal epidermal cell morphology before being considered suitable for analyzing the intracellular localization of GFP-AtEB1.

At low magnifications the GFP-AtEB1 label typically appeared as extending dots (Figure 2A; Movie 1 in the Supplemental Data). At higher magnifications the bright dots were seen to be the tips of faintly fluorescent filaments that appeared similar to those observed using a microtubule plus end-directed YFP-CLIP170 fusion protein [19]. The actual identity of these filaments was established by transient coexpression of the GFP-AtEB1 chimeric protein separately with either an YFP-MAP4 fusion protein that specifically labels microtubule arrays [20] or an YFP-mTalin fusion protein that labels F-actin microfilaments only [21]. Spectral separation and false color allocation to GFP and YFP signals was achieved

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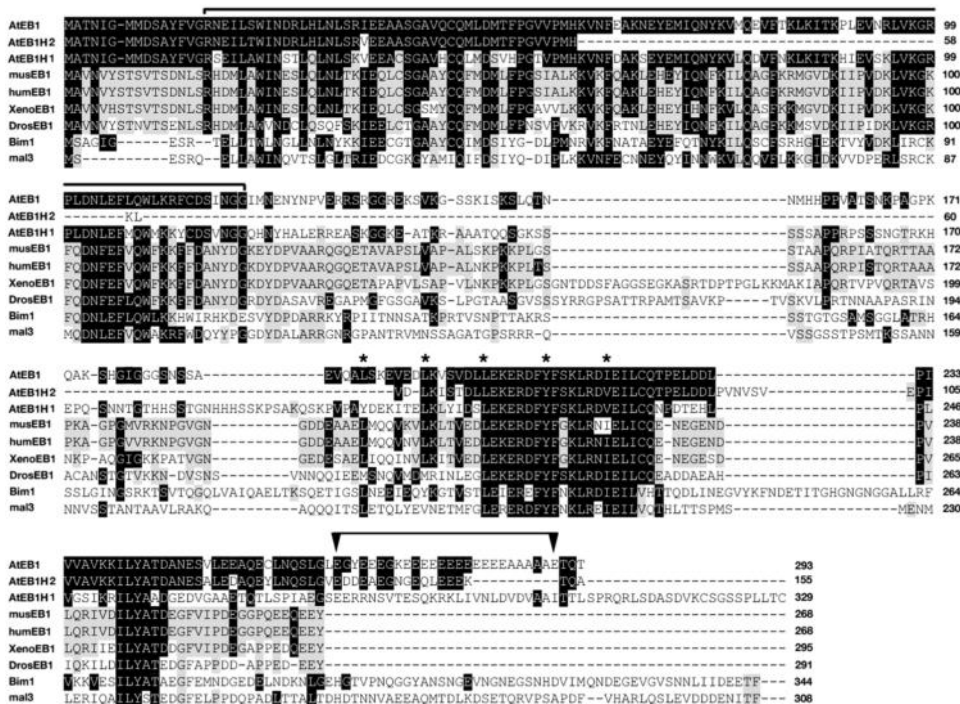


Figure 1. Protein Homology between EB1-Proteins from *Arabidopsis* and Other Organisms  
 AtEB1 (gi:10178081), AtEB1H2 (At3g47690; EMBL:AL049746), AtEB1H1 (At5g67270), HumEB1 (gi: 998356), XenoEB1 (gi:18478499), DrosEB1 (gi:18483681), Bim1 (gi:731441), and Mal3 (gi:1351729). Bracket extending over residues 16–121 denotes a conserved Calponin homology (CH) domain; conserved leucine residues (asterisks) suggest a leucine zipper motif. The C terminus of AtEB1 displays a long acidic tail of glutamic acid “E” residues (bracketed) that distinguishes it from other EB1 proteins.

using a spectrophotometric confocal imaging system (Leica TCS-SP2). As shown for the +TIP YFP-CLIP170 fusion protein in plant cells [19], the GFP-AtEB1 punctae clearly labeled the extreme 2–5  $\mu\text{m}$  growing (plus) ends of YFP-MAP4-labeled microtubules (Figures 2B–2E) and disappeared when the microtubule started shrinking (Figures 2B–2E). No positional correlation was found between the GFP-AtEB1 punctae and YFP-mTalin-labeled actin filaments (Figure 2F).

Studies on other EB1 family members have not revealed a cytoplasmic localization for the protein so far. However, all cell types in GFP-AtEB1 plants exhibited a novel cytoplasmic label in addition to the well-known microtubule plus end localization of the protein. The fluorescent GFP label localized to and outlined membranes surrounding large organelles, such as chloroplasts, mitochondria, and the nuclei, as well as motile, pleiomorphic membrane networks that rapidly interconverted between tubular forms and fenestrated lamellae (Figures 2G and 2H; Movie 3). A construct carrying GFP only was used as control for these observations and did not localize to either microtubules or endomembranes. Both localization patterns of AtEB1 were investigated further.

### Microtubule Localization of GFP-AtEB1 Reveals Its Targeting to Stabilized Regions of Microtubules

Two major microtubule-labeling patterns were observed in GFP-AtEB1 transgenic plants; one, where the green

fluorescence was limited to only 3–5  $\mu\text{m}$  of the microtubule tip and, two, where longer stretches (5–20  $\mu\text{m}$ ) of the microtubule were labeled green. Similar labeling patterns have been described using an YFP-CLIP170 fusion expressed in plant cells [19]. An analysis of cells at different stages of growth revealed that the punctate labeling of microtubule plus ends only was limited to actively growing/elongating cells, whereas ostensibly less active mature/interphase cells like those of fully expanded cotyledons exhibited the second, more extended pattern of microtubule labeling (Figures 3A and 3B). In other organisms, extensive EB1 labeling of microtubules has been interpreted as an artifact linked to its overexpression and leads to increased microtubule stability and decreased catastrophe events [3, 17]. However, neither the morphology nor the microtubule arrays in cells of GFP-AtEB1 plants suggested any signs of undue microtubule stabilization, indicating that the two patterns being observed were normal and not due to overexpression of the protein. An alternative explanation for the different patterns of microtubule labeling could then be that AtEB1 localizes to already stabilized regions of a microtubule. Two experiments were undertaken: one, that compared the microtubule labeling pattern of GFP-AtEB1 and a non-*Arabidopsis* EB1 to see if the former behaved differently and two, that artificially stabilized microtubules to observe changes in the GFP-AtEB1 localization on microtubules.

A mouse EB1-GFP that labels microtubule plus ends specifically but is known to label complete microtubules

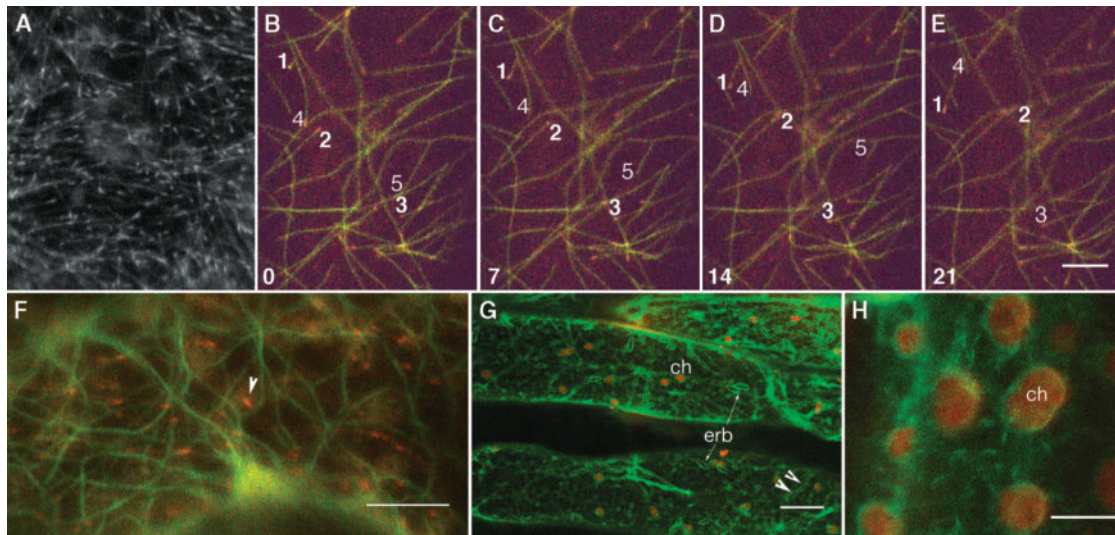


Figure 2. GFP-AtEB1 Exhibits a Dual Localization and Labels Microtubule Plus Ends and Endomembranes

(A) Punctate labeling observed in a leaf epidermal cell from GFP-AtEB1 transgenic *Arabidopsis* (Movie 1).

(B–E) A 21 s time-lapse series showing microtubules (labeled by YFP-MAP4 fusion protein and allocated a green color) and GFP-AtEB1 (allocated red color) colocalization. The merging of green-red labels produces a yellow color. Microtubules labeled 1, 2, and 3 all exhibit plus end growth with GFP-AtEB1 localized to the tip (yellow) whereas 4 and 5 exhibit retraction after losing the GFP-AtEB1 from their tips. Time in seconds given at the left side of each panel (Movie 2).

(F) An *Arabidopsis* epidermal cell coexpressing YFP-mTalin fusion-protein that labels F actin microfilaments (green) and GFP-AtEB1 (red) shows that the two probes do not colocalize.

(G) The cytoplasmic localization of AtEB1 highlights motile, singular tubular networks (arrowheads), as well as peripheral membranes of spindle-shaped ER bodies “erb” and diverse other organelles, including chloroplasts, “ch,” appearing red due to chlorophyll fluorescence (Movie 3).

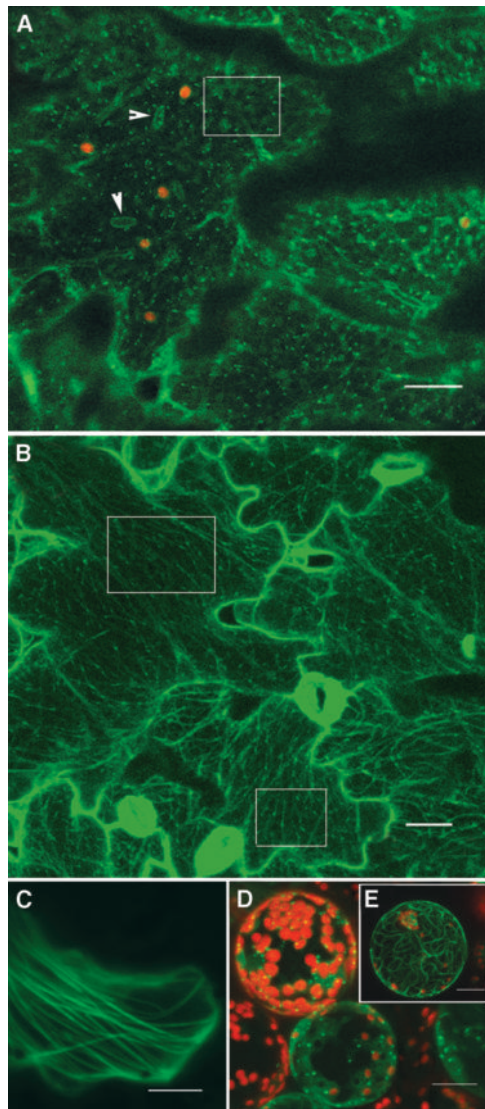
(H) Magnified view of a leaf cell showing the dual GFP-AtEB1 localization, which labels both peripheral chloroplast membranes ch and plus ends of microtubules (single green specks).

Scale bar in (G), 20  $\mu\text{m}$ ; all others, 8  $\mu\text{m}$ .

upon overexpression [3] was cloned under a plant-specific CaMV35S promoter. The mouse EB1-GFP and GFP-AtEB1 were independently expressed transiently in *Arabidopsis* plants.

Depending upon the cell type, reflecting probable differences in activity status, microtubules in cells expressing GFP-AtEB1 ( $n = 160$ ) showed localization patterns ranging from only plus end labeling to complete microtubule labeling (similar to Figures 3A and 3B) while their plus end extension rates ranged from 5 to 15  $\mu\text{m}/\text{min}$ . In contrast, microtubules were labeled completely (Figure 3C) in all cells expressing the mouse EB1-GFP ( $n = 98$ ) and were relatively static with less than 20% ( $n = 80$ ) showing plus end extension at the rate of  $3 \pm 1.3 \mu\text{m}/\text{min}$ . The observed labeling of complete microtubules by mouse EB1-GFP overexpression combined to their apparent bundling and reduced plus end growth rate suggested that as described for *Xenopus* A6 cells [3] overexpression of the mouse EB1 protein interferes with microtubule dynamics. However, a clear conclusion could not be drawn for AtEB1 since the transient assay merely confirmed our observation of differential labeling and microtubule plus end extension from stably transformed plants, indicating that these patterns reflected a normal situation for growing plant cells. Cells transiently expressing the mouse EB1-GFP or GFP-AtEB1 did not display morphological alterations, but whether the same holds true for plants stably expressing the mouse EB1-GFP remains to be investigated.

Artificial stabilization of microtubules was achieved through a treatment with the drug paclitaxel. Since even adjacent plant cells could show differences in the extent of microtubule labeling, we ensured homogeneity of paclitaxel treatment by using leaf protoplasts rather than complete plants. Within 10–20 min of adding 5  $\mu\text{M}$  paclitaxel to the protoplast culture medium, the GFP-AtEB1 label, initially limited only to the microtubule tips and therefore visible as motile punctae (Figure 3D), extended further until entire microtubules were highlighted within 30 min (Figure 3E). Control untreated protoplasts maintained the punctate labeling at microtubule plus ends. Microtubule plus end extension rates ( $n = 50$ ) of  $6 \pm 1.5 \mu\text{m}/\text{min}$  observed for untreated protoplasts were reduced to  $0.8 \pm 0.3 \mu\text{m}/\text{min}$  in paclitaxel-stabilized protoplasts. Taken together, our observations suggest that in *Arabidopsis*, while the extent of EB1 localization on a microtubule correlates with its dynamics, AtEB1 itself may not be responsible for stabilizing a microtubule. This is in contrast to the mouse EB1, which does stabilize and bundle microtubules in *Arabidopsis*. In addition, our drug experiments support the idea that AtEB1 localizes to already stabilized regions of microtubules. These observations appear consistent with the prevailing views on microtubule plus end dynamics where a GTP cap at the growing end plays an important role in governing the transition between growing and shrinkage. Removal of the GTP cap favors microtubule destabilization and leads to catastrophe [22], which is consis-



**Figure 3. The Extent of GFP-AtEB1 Localization on Microtubules Correlates with Changes in Microtubule Stability**

(A) The dual GFP-AtEB1 labeling in actively expanding cells of a cotyledon is visible as motile green dots at the plus ends of microtubules (e.g., punctae within outlined rectangle) and labile membrane tubules. Note the clear outlining of structures like ER-bodies (arrowheads) and chloroplasts (red).

(B) GFP-AtEB1 labeling of microtubules is more extensive (e.g., within rectangles) in mature cells of a fully expanded cotyledon.

(C) A single leaf epidermal cell transiently expressing a mouse EB1-GFP fusion protein shows complete labeling and apparent bundling of microtubules. Note that the punctae depicting microtubule plus ends (as seen in [A] and [B]) cannot be distinguished.

(D) Leaf protoplasts from GFP-AtEB1 transgenic plants show brightly fluorescent punctae denoting the plus ends of individual microtubules.

(E) A single GFP-AtEB1 leaf protoplast 15 min after adding 5  $\mu$ M paclitaxel to the protoplast culture medium where the labeling, initially limited to the microtubule tip (D), extended to highlight entire microtubules.

Scale bars: (A), (B), (D), (E), 20  $\mu$ m; (C), 8  $\mu$ m.

tent with the observation that all EB1 proteins typically target the relatively stable plus end but detach as soon as the end loses its stability and starts to shrink [1–3].

Our observation of extensive microtubule labeling by GFP-AtEB1 in cells at different developmental stages suggests that during development other microtubule-associated proteins may bind at different locations to enhance microtubule stability and thereby provide multiple lateral recruitment sites for AtEB1.

### Cytoplasmic Localization of GFP-AtEB1:

#### Targeting to Endomembranes

The general GFP-AtEB1 localization pattern suggested an association with endomembranes. A comparison was therefore made between the localization patterns of a DET3-GFP fusion protein, demonstrated to localize to endomembranes in plant cells [23] and GFP-AtEB1. Both probes produced an essentially similar membrane-labeling pattern. Moreover, a RFP (red fluorescent protein)-AtEB1 fusion was found to extensively colocalize with DET3-GFP (Figures 4A–4C) and confirmed the endomembrane localization of AtEB1.

Endomembrane localization of GFP-AtEB1 was also indicated in actively growing cells where the morphological plasticity and flow characteristics of GFP-AtEB1-labeled membranes were highly reminiscent of endoplasmic reticulum (ER) membranes and tubules, whereas a cortical ER-like mesh was conspicuously labeled in cotyledon epidermal cells (Figure 5A). In addition, GFP-AtEB1 outlined distinct,  $\sim 0.5$   $\mu$ m diameter, up to 5  $\mu$ m long, spindle-shaped motile organelles (Figures 2G and 3A) that resemble characteristic ER bodies present in members of the brassicaceae family of angiosperms, including *Arabidopsis thaliana* [24]. This suggested that the GFP-AtEB1 could also associate with the ER, a vital component of the endomembrane machinery. Further investigations involved covisualization of the ER and GFP-AtEB1 with the vital ER-tracker blue-white DPX (Figure 5B). In every cell type examined, the exterior of large ER structures, such as ER islands and ER bodies, was found encompassed by the GFP-AtEB1 label, while individual GFP-AtEB1-labeled membrane tubules frequently coalesced and merged with the existing ER (Movie 4). Further, we frequently observed microtubule plus ends moving along or as part of green fluorescent membranous tracks as they extended. These membrane tracks usually formed a part of the highly labile ER structure (Movie 4), suggesting that microtubules labeled with GFP-AtEB1 could pull along membranes that contribute to ER organization. In animal cells, +TIP complexes like CLIP170 have been shown to exhibit similar pulling along of membrane tubules to reorganize the endomembrane system in a microtubule polymerization-aided manner [7, 8]. Though belonging to the general +TIP group, association of EB1 family members to endomembranes has not been reported so far. In plants, though static electron microscopic images have loosely suggested microtubule associations with membrane tubules [25], this is the first time that dynamic membrane reorganization by a microtubule-associated protein has been actually observed.

However, in higher plants the motility of the endomembrane system has been shown to depend upon the actin cytoskeleton, though it is still unclear whether endomembrane organization also relies on the actin cytoskeleton [26]. Our observations on AtEB1 localization

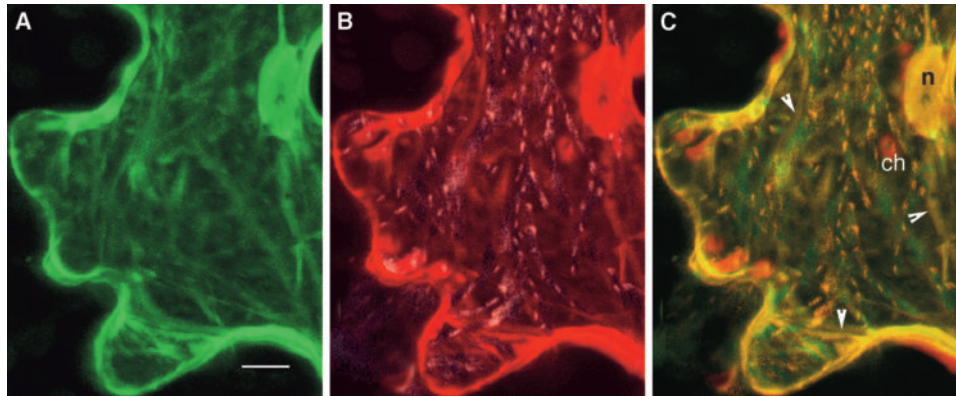


Figure 4. Confocal Images of a Single Leaf Epidermal Cell Coexpressing the Endomembrane-Localizing DET3-GFP Probe and RFP-AtEB1 (A) DET3-GFP localization in the green channel (spectral range 490–510) highlights only endomembranes and cytoplasmic strands. (B) RFP-AtEB1 localization in the red channel (spectral range 560–580) showing cytoplasmic membrane labeling as well as labeling of microtubule plus ends. (C) A merged image shows major areas of overlap (arrowheads) in the cytoplasmic labeling and around organelle periphery (e.g., nucleus, n). The bright red structures (ch) are chloroplasts. Scale bar, 10  $\mu\text{m}$ .

strongly linked microtubules to endomembrane organization. An interference with microtubule dynamics would thus be predicted to produce immediate changes in membrane organization but not necessarily its motility. It would be possible to visualize these changes with the GFP-AtEB1 probe. The next set of experiments tested this prediction by treating GFP-AtEB1 plants with actin- and microtubule-depolymerizing drugs.

#### Microtubule Depolymerization Increases Cytoplasmic Localization of GFP-AtEB1 and Alters Membrane Organization but Not Membrane Motility

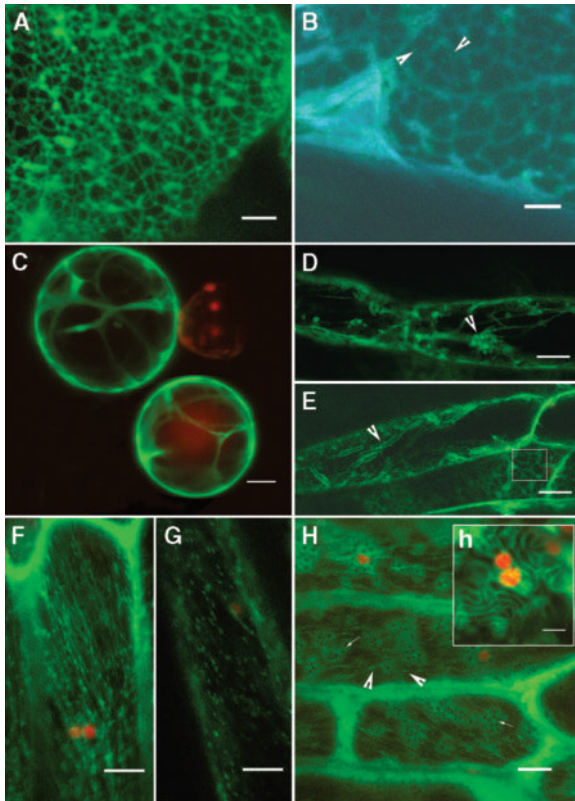
Leaf protoplasts from GFP-EB1 transgenic plants were treated with 2  $\mu\text{M}$  of the microtubule-depolymerizing drug oryzalin. Within 10 min of drug application most protoplasts had completely lost the microtubule plus end-associated GFP-EB1 label (Figures 5C and 5D) and instead exhibited a general green cytoplasmic fluorescence interspersed with numerous motile green globular bodies (Movie 5). Tubular-membrane structures normally labeled by the GFP-EB1 probe could not be detected either. Recovery of protoplasts after total microtubule depolymerization was slow and nonsynchronous, and therefore, seedlings of GFP-EB1 were also treated with oryzalin for 30 min. At the end of this period only motile, globular cytoplasmic aggregates could be seen in nearly 90% epidermal cells (Figure 5D). The seedlings were thoroughly washed for 10 min to remove the drug and then observed over 120 min for recovery of the microtubule cytoskeleton. At different time points, probably dependent on the severity of drug effect on different cells, the GFP-EB1 microtubule plus end label started appearing in cortical regions of the cell. Within 45 min, most epidermal cells had recovered sufficiently to exhibit microtubule plus end as well as membrane tubule labeling (Figure 5E). Control seedlings from a transgenic *Arabidopsis* line expressing a GFP-ER construct were treated similarly, and the ER was reduced to motile clumps within 30 min. These clumps recovered into the normal ER structure upon washing away the oryzalin (data not shown).

#### Actin Depolymerization Freezes Membrane Motility and Enhances GFP-AtEB1 Localization on Microtubules

A strong ER-actin connection has been established for plants [26], and therefore, GFP-AtEB1 plants were treated with the actin polymerization inhibitor latrunculin-B (LatB; 2  $\mu\text{M}$ ). The GFP-AtEB1 membrane labeling was maintained, but membrane flow completely ceased within 5 min of drug application in protoplasts and within 20 min in seedlings. Interestingly, in LatB-treated seedlings, the GFP-AtEB1 labeling of microtubules initially extended to highlight entire microtubules (Figure 5F compared to 5G), suggesting that actin depolymerization may either affect microtubule stability or alter the cytoplasmic and microtubule-associated distribution of AtEB1. However, LatB treatment at this concentration if continued for more than 45 min was detrimental to cell viability as it increased vacuolar compartments significantly and irreversibly altered membrane organization (Figure 5H). Microtubules lost their dynamicity and became short and static during the long LatB treatment. A similar observation of an initial ER freezing followed by its slow degradation was made for GFP-ER plants (data not shown). In all experiments, untreated controls maintained their usual localization patterns

#### Conclusions

The localization of AtEB1 to microtubule plus ends, its disappearance upon microtubule retraction, and its localization to endomembranes suggests that this protein shuttles between these intracellular destinations. The C-terminal acidic tail of AtEB1 distinguishes it from other family members and may be responsible for its cytoplasmic localization. AtEB1 behaves in a manner similar to that described for cytoplasmic linker proteins, like CLIP-170 in animal cells. Our results with AtEB1 also allow us to create a distinction between the motility and organization of endomembranes. We reaffirm that in higher plants membrane motility is actin dependent. However, the organization of membranes, including those of the ER and their polarized distribution, requires



**Figure 5. Identification of ER as One of the Cytoplasmic Structures Highlighted by GFP-AtEB1 and the Effect of Cytoskeletal Drugs on Membranes Organization and Motility**

(A) A compressed stack of 20 confocal sections (z step size, 1  $\mu\text{m}$ ) from an 8-day-old expanding cotyledon suggests that GFP-AtEB1 labels an ER-like structure.

(B) ER visualized using the vital ER tracker blue-white DPX was found to colocalize with the green fluorescent AtEB1 cytoplasmic localization, whereas the plus end-labeled microtubules were visible as single specks (arrowheads).

(C) GFP-AtEB1 leaf protoplasts exposed to 2  $\mu\text{M}$  of the microtubule depolymerizing herbicide oryzalin caused the microtubule plus end label of GFP-AtEB1 (compare with Figure 3D) to disappear within 10 min, leaving behind a general green-fluorescent cytoplasm.

(D) Hypocotyl epidermal cells of GFP-AtEB1 transgenics displayed numerous motile green globular bodies (arrowhead) that occupied the cell after oryzalin-induced depolymerization of microtubules, suggesting a change in membrane organization but not motility (Movie 5).

(E) Hypocotyl cells recovering from the oryzalin treatment after 45 min show the reemergence of the cytoplasmic and microtubule localization patterns of GFP-AtEB1. Note an ER-like localization pattern within the outlined rectangle and the presence of an ER body (arrowhead).

(F) Hypocotyl cells of GFP-AtEB1 transgenics exhibiting increased labeling of microtubules 15 min after treatment with 2  $\mu\text{M}$  of the actin polymerization inhibitor latrunculin B.

(G) Comparable cells from control untreated plants maintained the tip only labeling of microtubule plus ends.

(H) Hypocotyl cells exposed to 2  $\mu\text{M}$  LatB for 45 min show clumped up organelles (arrows) and short microtubules (arrowheads). (h) Characteristically nonmotile membrane folds seen in cells treated with LatB for long durations.

Scale bars: (A), (B), (C), 10  $\mu\text{m}$ ; (D), (E), (F), (G), (H), 8  $\mu\text{m}$ ; (h), 5  $\mu\text{m}$ .

an intact and functional microtubule cytoskeleton. This inference provides an explanation for the loss-of-polar-growth phenotype that emerges in plant cells with a compromised microtubule cytoskeleton. Accordingly, if site-directed deposition of membranes relies upon microtubule plus end dynamics for its execution, isotropic growth would be an inevitable consequence upon loss/compromised activity of microtubules. The EB1-microtubule-endomembrane interactions suggested by our observations may thus be vital for polarized cell development in higher plants.

#### Supplemental Data

Details of the experimental procedures, the biological materials, plasmids, and oligonucleotides used in this study, magnified views of Figures 5A and 5B, and Quicktime movies (1–5) showing AtEB1 behavior and localization pattern in living cells can be viewed online at <http://www.current-biology.com/cgi/content/full/13/22/DC1>.

#### Acknowledgments

We thank J. Olmsted for the human MAP4 clone; S. Craig for the mouse talin gene carrying the F actin binding domain; S. Tsukita and Y. Mimori-Kiyosue for the mouse EB1-GFP fusion construct; K. Schumacher for the DET3-GFP construct; and A. Mathur for help with computer imaging and movie creation. The work was supported by a Volkswagen Stiftung grant to M.H.

Received: July 3, 2003

Revised: September 1, 2003

Accepted: September 30, 2003

Published: November 11, 2003

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