MTV1 and MTV4 Encode Plant-Specific ENTH and ARF GAP Proteins That Mediate Clathrin-Dependent Trafficking of Vacuolar Cargo from the Trans-Golgi Network

Michael Sauer, a M. Otilia Delgadillo, a,1 Jan Zouhar, a,b,1 Gregory D. Reynolds, c Janice G. Pennington, d Liwen Jiang, e Sarah J. Liljegren, f York-Dieter Stierhof, g Geert De Jaeger, h,i Marisa S. Otegui, d Sebastian Y. Bednarek, c and Enrique Rojo, a,2

a Departamento Molecular de Plantas, Centro Nacional de Biotecnologia (Consejo Superior de Investigaciones Científicas), 28049 Madrid, Spain
b Centro de Biotecnologia y Genómica de Plantas, Universidad Politécnica, 28223 Madrid, Spain
c Department of Biochemistry, University of Madison, Madison, Wisconsin 53706
d Department of Botany, University of Madison, Madison, Wisconsin 53706
e School of Life Sciences, Centre for Cell and Developmental Biology, Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, China
f Department of Biology, University of Mississippi, Oxford, Mississippi 38677-1848
g Zentrum für Molekularbiologie der Pflanzen, University of Tübingen, 72076 Tuebingen, Germany
h Department of Plant Systems Biology, VIB, B-9052 Ghent, Belgium
i Department of Plant Biotechnology and Bioinformatics, Ghent University, B-9052 Ghent, Belgium

ORCID IDs: 0000-0001-9886-2917 (E.R.).

Many soluble proteins transit through the trans-Golgi network (TGN) and the prevacuolar compartment (PVC) en route to the vacuole, but our mechanistic understanding of this vential trafficking step in plants is limited. In particular, it is unknown whether clathrin-coated vesicles (CCVs) participate in this transport step. Through a screen for modified transport to the vacuole (mtv) mutants that secrete the vacuolar protein VAC2, we identified MTV1, which encodes an EPSIN N-TERMINAL HOMOLOGY protein, and MTV4, which encodes the ADP ribosylation factor GTPase-activating protein NEVERSHELDED/AGD5. MTV1 and NEV/AGD5 have overlapping expression patterns and interact genetically to transport vacuolar cargo and promote plant growth, but they have no apparent roles in protein secretion or endocytosis. MTV1 and NEV/AGD5 colocalize with clathrin at the TGN and are incorporated into CCVs. Importantly, mtv1 nev/agd5 double mutants show altered subcellular distribution of CCV cargo exported from the TGN. Moreover, MTV1 binds clathrin in vitro, and NEV/AGD5 associates in vivo with clathrin, directly linking these proteins to CCV formation. These results indicate that MTV1 and NEV/AGD5 are key effectors for CCV-mediated trafficking of vacuolar proteins from the TGN to the PVC in plants.

INTRODUCTION

Intracellular compartmentalization and multicellular development are two evolutionary innovations of pivotal importance for understanding the basic biology of many eukaryotic organisms, including all metazoa and land plants. An endomembrane system of near modern complexity may have been present in the last common eukaryotic ancestor (Dacks and Field, 2007). Indeed, most of the proteins responsible for trafficking are conserved throughout all eukaryotes, although they tend to be expanded in number in multicellular organisms (Dacks and Field, 2007; Sanderfoot, 2007). By contrast, multicellularity is not universal in eukaryotes and is thought to have evolved independently in plants and animals (Meyerowitz, 2002). However, the main model system for studying intracellular trafficking has been the unicellular yeast Saccharomyces cerevisiae, and so many of the unique features present in multicellular models remain relatively unexplored. In plants, the specific constraints imposed by multicellularity and their distinct developmental strategy have led to specific arrangements of the endomembrane system. A conspicuous peculiarity of the plant endomembrane system is the presence of large vacuoles, which in most plant cells occupy the majority of the cellular volume. The enlarged vacuoles of plants are necessary adaptations to autotrophy and immobility, as they provide an energy-efficient organ expansion mechanism for exploring the surroundings and a buffering organelle to maintain cellular homeostasis (Zouhar and Rojo, 2009). Plants may contain different types of vacuoles in a single cell, and they develop specialized vacuoles in certain cells, such as the protein storage vacuoles in seed tissues (Rojo and Denecke, 2008), which constitute the main source of proteins for human and livestock nutrition.
Despite their importance, the molecular machinery responsible for the delivery of proteins and membrane to these distinct vacuoles is largely uncharacterized. Two different types of vesicles, clathrin-coated vesicles (CCVs) and dense vesicles, bud from the trans-Golgi network (TGN) and contain vacuolar cargo in plants (Otegui et al., 2006; Hinz et al., 2007; Kang et al., 2011), but their actual contribution to vacuolar trafficking remains unresolved. Moreover, a vesicle-independent compartmental maturation model for vectorial transport from the TGN to the prevacuolar compartment (PVC) has been recently proposed (Scheuring et al., 2011). To test the validity of these models and attain a mechanistic understanding of vacuolar transport in plants, it is essential to identify the trafficking machinery involved in the different steps of the pathway.

In S. cerevisiae, genetic analysis has been instrumental in revealing the role of many proteins in intracellular trafficking and in understanding the organization and function of the endomembrane system in this unicellular organism (Schekman and Novick, 2004). In comparison, our knowledge of the increased repertoire of trafficking components in multicellular eukaryotes is limited, in part because genetic dissection of intracellular trafficking is not as straightforward as in yeast. In plants, transient transport assays and, more recently, genetic analysis, have been used for studying vacuolar trafficking (Denecke et al., 2012). Through these combined efforts, several factors involved in vacuolar transport in plants have been characterized (Rojo and Denecke, 2008); in particular, the function of the vacuolar sorting receptors (VSRs) in this pathway is relatively well understood (De Marcos Lousa et al., 2012). However, the number of vacuolar trafficking components identified is still more than one order of magnitude lower than those known to function in this process in yeast (Bonangelino et al., 2002; Ricarte et al., 2011), and it is therefore evident that we still lack most information on the core functioning of the vacuolar pathway in plants. In particular, the machinery involved in anterograde trafficking from the TGN is uncharacterized, and it is unknown whether vesicle trafficking participates in this step.

Vesicular transport of proteins involves cargo recruitment to specific membrane domains followed by induction of vesicle budding and cargo loading into the incipient vesicles. VSRs are a plant-specific family of type I transmembrane proteins that recruit soluble vacuolar cargo through direct protein–protein interaction and have been shown genetically to be necessary for transport of the cargo to the vacuole (Shimada et al., 2003; Zouhar et al., 2010). VSRs cycle between the TGN and the PVC (Sanderfoot et al., 1998; Tse et al., 2004; Reichardt et al., 2007; Foresti et al., 2010; Saint-Jean et al., 2010), interact in vitro with μ-adaptins (Sanderfoot et al., 1998; Happel et al., 2004), and are found in CCV-enriched fractions (Hinz et al., 1999), indicating that they may mediate sorting and loading of soluble cargo into CCVs for their transport to the vacuole (De Marcos Lousa et al., 2012). However, since TGN factors coupling VSR cargo recruitment to CCV budding and cargo loading into the vesicles are unknown, this trafficking model remains speculative. It has been reported that Arabidopsis thaliana Epsin1 binds clathrin and VSR1 and has a role in trafficking of a chimeric vacuolar cargo (Song et al., 2006a); however, Epsin1 localization at the TGN or an in vivo role in VSR cycling and in trafficking of endogenous vacuolar proteins has not been documented. Epsin1 is one of 43 Arabidopsis EPSIN N-TERMINAL HOMOLOGY (ENTH) proteins that are characterized by a conserved phospholipid binding ENTH domain for insertion into membranes. ENTH proteins contain in many cases clathrin binding motifs (Legendre-Guillemin et al., 2004) that allow them to function as monomeric adaptors for clathrin coat recruitment to membranes (Horvath et al., 2007).

Another class of proteins that has been shown to bind clathrin in animal systems is the ADP ribosylation factor GTase-activating protein (ARF GAP) family, whose members induce the hydrolysis of GTP bound to ARF and are essential factors to couple vesicle formation with cargo loading (Tanabe et al., 2005; Natsume et al., 2006; Spang et al., 2010; Bai et al., 2011). However, there are no prior reports of plant ARF GAPs binding to clathrin or having a role in vacuolar trafficking. Through a genetic screen, we identified the MODIFIED TRANSPORT TO THE VACUOLE1 (MTV1) and MTV4 Arabidopsis genes, which encode plant-specific members of the ENTH and ARF GAP protein families localized at the TGN and in CCVs. MTV1 and MTV4 bind clathrin and cooperatively participate in the transport of vacuolar cargo and VSRs, suggesting that they are key effectors coupling VSR-dependent cargo recruitment to cargo loading into CCVs for vectorial transport from the TGN to the PVC.

RESULTS

MTV1 and MTV4 Encode Plant-Specific Proteins with ENTH and ARF GAP Domains, Respectively

To identify components required for vacuolar trafficking of soluble cargo, we conducted a mutant screen, which has been previously described in more detail (Sanmartín et al., 2007). Briefly, a dodcapeptide derived from the CLAVATA3 (CLV3) protein is the extracellular ligand of the CLV receptor kinase complexes (Betsuyaku et al., 2011). Via this signaling pathway, CLV3 negatively regulates the activity of WUSCHEL, thus reducing the stem cell pool size in the shoot apical meristem. For the screen, CLV3 was fused to the vacuolar sorting signal of barley (Hordeum vulgare) lectin and expressed under the 3SS promoter, creating a chimeric protein termed VAC2. VAC2 is recognized by the vacuolar sorting machinery and transported to the vacuole, which prevents its secretion into the extracellular space and effectively renders it inactive (Rojo et al., 2002). Mutations that disrupt vacuolar trafficking may result in VAC2 export into the extracellular space via the default secretion pathway. There, VAC2 activates the CLAVATA pathway and leads to meristem consumption (Rojo et al., 2002; Sanmartín et al. 2007). We used this highly sensitive assay to screen for mtv mutants that secrete VAC2 into the extracellular space, resulting in premature termination of the shoot apical meristem (Figure 1A).

We isolated two mtv mutants from an ethyl methanesulfonate (EMS)-mutagenized VAC2 population, termed mtv1-1 and mtv4-1. Both mutants exhibit determinate growth of shoot apical meristems (Figure 1B) and premature termination of floral meristems, which in most cases did not produce carpels. These meristem
phenotypes were dependent on the presence of the VAC2 trans-gene, indicating that they are due to mis-secretion of VAC2 and not to a direct activity of MTV1 and MTV4 on meristems. We identified the mutant loci by a map-based cloning strategy. The mutation in mtv1-1 was mapped to a region in chromosome 3, containing 24 genes (At3g16180 to At3g16410), and by sequencing of candidate genes we discovered a nonsense mutation in the coding sequence of the At3g16270 locus. The mtv1-1 mutation introduces a stop codon after only 11 amino acids of the protein sequence and is thus predicted to be a null allele (Figure 1C). At3g16270 had not been functionally characterized yet and is annotated in the TAIR10 database as an ENTH domain containing a protein of unknown function.

MTV4 was mapped to a region on chromosome 5 containing 59 genes (At5g54160 to At5g54630). Sequencing of candidate genes revealed a nonsense mutation in the coding sequence of the At5g54310 locus, converting Trp-76 to a premature stop. At5g54310 has been previously identified as NEVERSHED/ARF-GAP5 (NEV/AGD5) (Liljegren et al., 2009). mtv4-1 plants displayed floral organ abscission defects characteristic of the nev mutant phenotype and thus represent a novel allele of nev/agd5, which we designated as agd5-10 (Figure 1C).

To verify that the genes identified were the causative loci for the terminated meristem phenotype in the VAC2 background, we crossed the VAC2 reporter line with T-DNA insertion mutants mtv1-2 (Salk_061811) and mtv4-2/nev-7/agd5-7 (Salk_079928), both of which harbor insertions in the first intron of the respective genes (Figure 1C). In the F2 of these crosses, the meristem termination phenotype was recapitulated in homozygous mutant plants containing the VAC2 transgene, confirming that the mutations in MTV1/At3g16270 and NEV/AGD5/MTV4 indeed cause secretion of the VAC2 marker (see Supplemental Figure 1A online). Immunoblot analysis showed that both the original EMS alleles and the T-DNA alleles are full loss-of-function mutants of MTV1 and NEV (see Supplemental Figure 1B online). For all subsequent analyses described in this article, we used the respective T-DNA alleles, unless otherwise stated.

MTV1 has not been characterized previously. Analysis of the primary amino acid sequence indicated that the predicted MTV1 protein contains only one identifiable conserved domain, an N-terminal (amino acids 1 to 134) ENTH/VHS domain (InterPro code IPR008942). Interestingly, an NMR spectrum of MTV1’s ENTH domain has been generated (López-Méndez et al., 2004), which structurally confirms its classification. Although this domain is predicted to be encoded in 43 other Arabidopsis proteins, WU-BLAST analysis showed that full-length MTV1 did not have any significant homology to any other protein in the TAIR10 protein database. However, a search for orthologs in other plant species indicated that MTV1 is conserved throughout the angiosperms. We performed an analysis of protein identity/similarity to Arabidopsis MTV1 and found values between 95.2 and 50.9% identity in the analyzed members of the angiosperm clade (Figure 1D; see Supplemental Data Set 1 online). Notably, in Selaginella and Physcomitrella, we found orthologs with 36.8% identity and 54.1% similarity, suggesting that MTV1 may be conserved also outside of the spermatophytes. Furthermore, in most plant species, we could identify only one ortholog of MTV1, indicating that it is a well conserved and mostly single-copy gene.
A phylogenetic analysis of orthologs of MTV1, Epsin1, Epsin2/2R, and Epsin3, clearly separates the genes into three distinct clades (see Supplemental Figure 2 and Supplemental Data Set 2 online). Taken together, MTV1 represents a novel and unique member of the group of ENTH domain containing proteins, which is potentially involved in vacuolar protein trafficking.

MTV4/NEV/AGD5 encodes a member of the ARF GAP protein family, which in Arabidopsis contains 15 members (Vernoud et al., 2003). Interestingly, in contrast with the other 14 Arabidopsis ARF GAPS, the homology of NEV/AGD5 with proteins outside the plant kingdom is restricted to the ARF GAP domain (amino acids 16 to 130), whereas in plant species, there are proteins sharing identity throughout the whole sequence with NEV/AGD5 (amino acids 1 to 483), suggesting that these latter form a clade of plant-specific ARF GAPS (Liljegren et al., 2009; this article).

**MTV1 and NEV/AGD5 Exhibit Near-I dentical Patterns of Expression and Interact Genetically**

Previous functional analyses showed that NEV/AGD5 is required for correct abscission of floral organs, which is a specific developmental process restricted to a limited number of cells (Liljegren et al., 2009). However, our result demonstrating the secretion of VAC2 and the resulting shoot apical meristem termination in the nev/agd5 mutant points toward a more general role of NEV/AGD5 in trafficking events. Previous tissue-specific analyses of NEV/AGD5 expression revealed broad expression in flowers, leaves, stems, and roots (Liljegren et al., 2009).

To further define the tissues and developmental stages in which NEV/AGD5 could play a role, we generated stable pAGD5:AGD5-GUS (for β-glucuronidase) transgenic lines using an additional upstream regulatory sequence and analyzed tissues of different developmental stages. We detected GUS signal in almost all tissues of the plant (Figure 2A). Strong signal was observed in the root meristem, root, shoot, and leaf vasculature with less expression in leaf ground tissue (Figure 2A, i to iii and vii). Importantly, we observed strong signal in developing and mature embryos (Figure 2A, iv–vi), which would be consistent with a role in the transport of storage proteins to protein storage vacuoles, which peaks during mid and late stages of embryogenesis (Santos Mendoza et al., 2008). Intriguingly, pMTV1:MTV1-GUS transgenic lines exhibit a nearly identical expression pattern (Figure 2B), although MTV1 expression was absent from the floral abscission zone (cf. Figures 2A, vii, and 2B, vii, arrowheads). Based on these expression data and the virtually identical meristem termination phenotype when in the VAC2 background, it appears possible that MTV1 and NEV/AGD5 act in a common trafficking pathway.

To test the hypothesis that MTV1 and NEV/AGD5 have associated functions, we analyzed the functional consequences of combining the mvt1 and nev/agd5 mutations. After crossing the single mutants, we noted in the segregating F2 population the occurrence of severely dwarfed plants (Figures 2C and 2D). These were subsequently identified as mvt1 nev/agd5 double homozygotes by genotyping. Double mvt1 nev/agd5 mutants have reduced growth of both aerial and underground organs (Figures 2C and 2D). However, despite the drastic reduction in vegetative plant size, they have a relatively normal development and show no obvious patterning defects. Interestingly, flowers were the only organs of normal size in the double mutant (Figure 2F), which may be due to higher expression levels of genes with redundant functions (i.e., other ENTH or AGD proteins) in those organs. Pollen viability was not affected, as demonstrated by vital Alexander stain (Figure 2E), but the siliques of the double mutant were consistently shorter than those of the wild type or single mutants and exhibited the same abscission defect as observed in the nev/agd5 single mutants (Figure 2G, arrowheads; Liljegren et al., 2009). As expected, the reduced plant stature, smaller number of siliques, and reduced siliqu e size drastically reduce total seed yield in the double mutants. The synergistic genetic interaction observed suggests a functional relationship (Pérez-Pérez et al., 2009) and that MTV1 and NEV/AGD5 converge at a trafficking node that is essential for proper organ and plant growth. This agrees with prior reports showing similar synergistic impairment of plant growth when combining mutations in homologous, redundant trafficking genes (Surpin et al., 2003; Zhang et al., 2007; Uemura et al., 2012) or mutations in nonhomologous trafficking genes that encode components of a protein complex (Ebine et al., 2008).

**MTV1 and NEV/AGD5 Function Specifically in the Transport of Vacuolar Cargo**

The finding that MTV1 and MTV4 encode ENTH and ARF GAP proteins suggests that VAC2 secretion in the respective mutants is due to a block in vesicular trafficking and implies that endogenous cargo using the same pathway should also be affected. 12S globulins and Aleurain are endogenous Arabidopsis vacuolar proteins that are secreted in other mutants affected in VAC2 trafficking, suggesting that they share a common trafficking pathway (Sanmartín et al., 2007; Zouhar et al., 2009, 2010). Importantly, the ubiquitous expression of MTV1 and NEV/AGD5 in seeds and in vegetative tissues is compatible with a role in 12S globulins and Aleurain trafficking. Processing of 12S globulins occurs between the PVC and the vacuole (Otegui et al., 2006), so the accumulation of precursor forms of larger size is indicative of defects in reaching these compartments (Shimada et al., 2003; Li et al., 2006). To test whether MTV1 and NEV/AGD5 are involved in 12S globulin transport, we compared the protein profile of wild-type and mutant seeds. In nev/agd5 and mvt1 single mutants, we detected a slight increase in the accumulation of ~50-kD precursor forms of 12S globulins, which became pronounced in the double mvt1 nev/agd5 mutant (Figure 3A). Consistent with a reduced transport of these major storage proteins to the vacuole, microscopy analysis showed that both singles and the double mutant had smaller, more rounded PSVs with larger spaces in between (Figures 3B and 3C), indicative of defects in PSV filling during seed biogenesis. These results suggest that NEV/AGD5 and MTV1 cooperate in the trafficking of 12S globulins to the vacuole, likely at a stage prior to the transport of 12S globulin precursors from the TGN to the PVC.

To determine whether NEV/AGD5 and MTV1 also affect transport of endogenous vacuolar cargo in vegetative tissues, we tested for mis-secretion of the vacuolar protease Aleurain (Ahmed et al., 2000). We analyzed the levels of Aleurain in
extracellular fluid fractions from fully expanded leaves, contrasting them with the levels of actin, the V-ATPase subunit DET3, and the endoplasmic reticulum chaperone BiP as controls for contamination from intracellular proteins during extraction. In fractions from wild-type and nev/agd5 plants, we found trace amounts of Aleurain, actin, and DET3 and the absence of BiP (Figure 3D). Importantly, a moderate but specific increase in Aleurain levels in the extracellular fraction was observed in mtv1 plants, which was pronounced in the mtv1 nev/agd5 double mutants. Moreover, a precursor form of Aleurain was specifically found in the extracellular fluid fraction from mtv1 and mtv1 nev/agd5 plants, consistent with prior data showing incomplete processing of Aleurain when it is secreted into the apoplasm (Zouhar et al., 2010). To gain additional evidence for abnormal secretion of vacuolar cargo in the mutants, we used an antibody against PR5, which has been found in the vacuole proteome of Arabidopsis leaves (Carter et al., 2004). Similar to Aleurain, we found increased accumulation of PR5 in the apoplasm of mtv1 and mtv1 nev/agd5 leaves. To rule out that the higher levels of Aleurain and PR5 in the extracellular fractions were due to a potential upregulation of PR5 and Aleurain expression or increased protein stability, we also compared total protein extracts from the same leaf material. We did not find increased protein amounts in the mutants (see Supplemental Figure 3 online), indicating that PR5 and Aleurain accumulation in the

Figure 2. Expression and Phenotypic Characterization of mtv1 and nev/agd5 Single and Double Mutants.

(A) Expression of NEV/AGD5 as revealed by the ProAGD5:AGD5-GUS transgene.
(B) Expression of MTV1 as revealed by the ProMTV1:MTV1-GUS transgene. Lowercase roman numerals indicate tissue: (i) primary seedling root, (ii) 11 DAG plant, (iii) expanded leaf of soil-grown plant, (iv) immature seed at heart embryo stage, (v) heart stage embryo, (vi) mature embryo, (vii) inflorescence with flowers of different developmental stages; arrowhead marks abscission zone.
(C) Phenotype of 4-week-old wild-type, nev/agd5, mtv1, and nev/agd5 mtv1 double mutant plants. A magnified image of the boxed nev/agd5 mtv1 plant is shown below.
(D) Root length of 5 DAG seedlings, as a box plot; whiskers indicate population range; n > 40.
(E) Pollen viability is not altered in the mtv1 nev/agd5 double mutant as indicated by Alexander stain.
(F) Flower size is not altered in the mtv1 nev/agd5 double mutant.
(G) Mature siliques. Arrowheads mark nonabscised floral organs in nev/agd5 single and mtv1 nev/agd5 double mutants. Bars = 100 µm in (i) and (vi), 2 cm in (ii) and (iii), 25 µm in (iv), (v), and (E).
Figure 3. Mutations in mtv1 and nev/agd5 Specifically Affect Vacuolar Transport of Endogenous Proteins and Do Not Alter Endocytosis, Polar Targeting, Endosomal Recycling, or Secretion.

(A) Immunoblot analysis of seed protein extracts with 12S globulin antibody; arrowhead indicates the 52-kD precursor (p).

(B) Autofluorescence of protein storage vacuoles (PSVs) in hypocotyls of mature embryos from dry seeds.

(C) Size distribution of PSVs from each genotype is displayed in a bar graph (number of PSVs measured: n = 470 for Col-0, n = 566 for mtv1, n = 661 for agd5, and n = 660 for mtv1agd5). The mean PSV size (X) of the different genotypes is shown above the arrowheads. The PSVs were significantly smaller in mtv1 (X = 14.0 µm², P = 4.4 x 10⁻⁶, unpaired t test), agd5 (X = 11.4 µm², P = 1.4 x 10⁻¹⁶, unpaired t test), and mtv1agd5 (X = 11.6 µm², P = 1.6 x 10⁻¹⁵, unpaired t test) than in wild-type embryos (X = 16.7 µm²).

(D) Immunoblot analysis of extracellular fluid from fully expanded leaves of wild-type, single, and double mutants. ALEU, Aleurain proteinase; ALEU ov. ex., same as above but with increased exposure time to reveal an unprocessed precursor band (p, precursor form; m, mature form); PR5, thaumatin-like protein; DET3, subunit C of the V-ATPase; BiP, luminal binding protein BiP; Ponceau loading control.

(E) FM4-64 as marker for endocytic uptake. The double mutant shows similar levels of intracellular (i.e., endocytosed) signal.

(F) to (H) PIN2-GFP signal (green) in propidium iodide (red) counterstained root epidermal cells.

(F) PIN2-GFP localizes to the apical polar domain of the wild type and the double mutant.

(G) PIN2-GFP is incorporated into BFA bodies after 1 h of BFA treatment in the wild type and double mutant.

(H) PIN2-GFP relocates to the apical plasma membrane 2 h after BFA was washed out in the wild type and the mutant.

(I) The secretory marker SP-RFP is secreted to the extracellular space of cotyledon epidermal cells in the wild type and the double mutant. Bars = 10 µm.
extracellular fluid of the mutant leaves is indeed caused by the specific mistargeting of vacuolar cargo.

To explore whether the mtv1 and nev/agd5 mutations affect other trafficking pathways, we analyzed the fate of endocytic and secretory markers in the mtv1 nev/agd5 double mutant, where vacuolar phenotypes were most pronounced. We used the lipophilic styryl dye FM 4-64, which first labels the plasma membrane, is then internalized via endocytosis, passes through the endosomes, and ultimately reaches the tonoplast membrane (Bolte et al., 2004; Dettmer et al., 2006). We did not detect any changes in the dynamics of uptake and transit through the endosomes of FM4-64 in the roots of double mutants (Figure 3E; see Supplemental Figure 4 online). As an additional probe for endocytosis, we analyzed the internalization of the PIN1 and PIN2 auxin transporters in wild-type and double mutant roots. These transporters constitutively cycle between the plasma membrane and early endosomes, but treatment with brefeldin A (BFA), which inhibits the exocytotic step of recycling, leads to PIN accumulation in BFA bodies (Geldner et al., 2003; Langhans et al., 2011). We did not find any difference in BFA body accumulation of PIN1 and PIN2 between the wild type and double mutant (Figures 3F and 3G; see Supplemental Figures 5A and 5B online), suggesting that NEV/AGD5 and MTV1 do not play a role in these endocytic events. On the other hand, once BFA is removed, the endocytosed material is recycled from the BFA body to the plasma membrane in an exocytotic process similar to secretion. We performed BFA washout experiments with PIN1-GFP (for green fluorescent protein) and PIN2-GFP and found no changes between the wild-type and double mutant plants (Figure 3H; see Supplemental Figure 5C online), indicating that exocytotic processes were not affected. In these experiments, we also did not observe any defects in the polar targeting of PIN1-GFP or PIN2-GFP in the double mutant (Figures 3F and 3H), further corroborating that endo- and exocytosis, which govern PIN polarity (Geldner et al., 2003; Kleine-Vehn et al., 2008), are not dependent on the function of MTV1 or NEV/AGD5. As an additional test for secretion of a nonmembrane protein, we observed the secretory marker SP-RFP (for red fluorescent protein) (Hunter et al., 2007). We invariably detected it in the extracellular space of cotyledon epidermal cells of either the wild type or double mutants (Figure 3I). Taken together, our results suggest that MTV1 and NEV/AGD5 function specifically in vacuolar trafficking. Moreover, the synergistic phenotype of the double mutant indicates that MTV1 and NEV/AGD5 cooperate in a common trafficking step and the fact that secretion is not affected suggests that this is a post-Golgi step specific for the vacuolar pathway.

MTV1 and NEV/AGD5 Colocalize at the TGN and Redistribute to the Periphery of BFA Bodies

NEV/AGD5 has been shown to colocalize with bona fide TGN-associated proteins, such as VT112 (by communolocalization, Liljegren et al., 2009) and SYP61 (by transient coexpression assays in tobacco (Nicotiana tabacum) leaf cells; Stefano et al., 2010), suggesting that NEV/AGD5 may regulate ARF-dependent formation of specific vesicles at the TGN. By contrast, the localization of MTV1 is unknown. To study the subcellular distribution of MTV1, we stably transformed plants with a prMTV1:MTV1-GFP fusion construct and observed its subcellular localization by in vivo confocal imaging in root epidermal cells. This construct complements the mtv1 mutant, suggesting that it reproduces the activity of the endogenous gene. MTV1-GFP signal was found in punctate structures distributed broadly throughout the cytosol, which indicates that MTV1 resides at or is associated with an endomembrane compartment.

To define this compartment further, we crossed prMTV1:MTV1-GFP plants with a range of previously described fluorescent marker lines for various endomembrane compartment types. The LE/PVC marker wave2R (RabF2b/ARA7) (Geldner et al., 2009) did not show significant colocalization with MTV1 (Figure 4A). The phosphatidylinositol-3 kinase inhibitor wortmannin leads to swelling of the PVC into characteristic ring-like structures and is frequently used to identify this type of compartment (Tse et al., 2004; Wang et al., 2009). After wortmannin treatment, we observed wave2R-labeled ring-like structures, whereas MTV1-GFP fluorescence remained in a punctate pattern (Figure 4B, arrowheads), strongly suggesting that MTV1 is not associated with the PVC. Next we tested the Golgi marker wave22R (SYP32) (Geldner et al., 2009), and although there was no overlap with MTV1-GFP, the signals were frequently found in close proximity (Figure 4C; see Supplemental Figures 6A and 6B online). Time-lapse imaging showed that MTV1 comigrates with the Golgi marker (see Supplemental Movie 1 and Supplemental Figure 7A online), indicating that MTV1 likely resides on a Golgi-associated structure, such as the TGN (Viotti et al., 2010). Therefore, we crossed MTV1-GFP with the TGN markers SYP61-CFP (for cyan fluorescent protein; Robert et al., 2008) and VHA-a1-RFP (Dettmer et al., 2006) and found a very high degree of overlap and a nearly perfect comigration in time-lapse imaging (Figures 5A and 5B; see Supplemental Figures 6C, 6D, and 7B and Supplemental Movie 2 online), indicating that MTV1 localizes primarily to the TGN. The small fraction of MTV1 signal that comigrates but does not overlap with the signal from TGN markers may represent partial localization of the protein in trans-Golgi cisternae. Moreover, we observed strong colocalization of the MTV1-GFP–labeled structures with FM4-64 after short incubation times (Figure 5C, arrowheads), when the internalized dye has only reached the TGN (Dettmer et al., 2006; Gendre et al., 2011). By contrast, we could not detect any MTV1-GFP at the plasma membrane colocalizing with FM4-64 (Figure 5C, open arrows) or at the cell plate (Figure 5D) colocalizing with wave129R (RabA1g; Geldner et al., 2009). These results suggest that MTV1 resides at the TGN, as does NEV/AGD5, an observation that is consistent with their genetic and functional interaction. To test for their colocalization at the TGN directly, we generated an AGD5-RFP fusion and crossed it into plants carrying MTV1-GFP. We observed a strong degree of overlap, confirming that MTV1 and AGD5 reside on the same TGN compartment (Figure 5E; see Supplemental Figures 6E and 6F online). To further refine the localization of MTV1 at the ultrastructural level, we performed immunogold labeling on MTV1-GFP root cells using anti-GFP antibody and quantitatively analyzed gold particle localization. We observed the highest density of gold particles associated with the TGN (Figure 5F, arrowheads, and 5G; see Supplemental Figure 8 and Supplemental Table 1 online), confirming the results obtained by confocal light microscopy.
In Arabidopsis roots, treatment with the trafficking inhibitor BFA results in the redistribution of the TGN into aggregates called BFA bodies (Langhans et al., 2011). Surprisingly, we found that MTV1, while generally responsive to BFA, did not localize to the core of the BFA bodies, where TGN markers such as SYP61-CFP, VHA-a1-RFP, or endocytosed FM4-64 are found (Figures 6A and 6B; Dettmer et al., 2006; Lam et al., 2009). Instead, MTV1 was found in the outer periphery of the BFA body (Figures 6A and 6B). An apparently similar response to BFA was reported for cis-Golgi markers, such as wave22R (SYP32) (Geldner et al., 2009). However, we found that the peripheral SYP32-RFP– and MTV1-GFP–labeled structures after BFA treatment were practically mutually exclusive (Figure 6C), indicating that the peripheral BFA body localization of MTV1 does not correspond to the cis-Golgi. Importantly, NEV/AGD5 also exhibited this peculiar response to BFA treatment (Figure 6D). Taken together, our results demonstrate that MTV1 and NEV/AGD5 colocalize at the TGN, possibly in a subdomain characterized by its peripheral BFA body distribution.

MTV1 and NEV/AGD5 Function in Clathrin-Dependent Trafficking from the TGN

The presence of ARF GAP and ENTH domains in NEV/AGD5 and MTV1 suggests that these proteins may participate in vesicle formation. In particular, they could be involved in the biogenesis of CCVs that bud from the TGN in Arabidopsis and contain Aleurain (Otegui et al., 2006; Hinz et al., 2007). Interestingly, the TGN localization of MTV1 and NEV/AGD5 and the peculiar redistribution in response to BFA is very similar to that reported for clathrin (Ito et al., 2012). To directly assay for colocalization with clathrin, we analyzed plants expressing MTV1-mCherry and clathrin light chain-GFP (CLC-GFP). We observed a high degree of overlap between CLC-GFP and MTV1-RFP signals (Figure 7A), in line with the reported major localization of CLC to the TGN (Ito et al., 2012). Importantly, after treatment with BFA, CLC-GFP and MTV1-RFP were found together in the peripheral zone of the BFA bodies (Figure 7B). We conclude from these analyses that MTV1, NEV/AGD5, and CLC colocalize at the TGN, where MTV1 and NEV/AGD5 may participate in CCV formation. To determine whether NEV/AGD5 and MTV1 are associated with CCVs, we purified CCVs from Arabidopsis suspension culture cells. Electron microscopy analysis of the samples demonstrates that CCVs were efficiently isolated to high purity (Figure 7C). In protein gel blot analyses, we found very strong enrichment of clathrin heavy chain (CHC) in the CCV fraction and a corresponding depletion of markers from other cellular compartments, confirming a high level of purification (Figure 7D).
Figure 5. MTV1 and NEV/AGD5 Localize to the Early Endosome/TGN.
Importantly, NEV/AGD5 and MTV1 were also highly enriched in the CCV fraction, in line with their suspected role in CCV formation. To further corroborate this result, we performed immunogold labeling on the isolated CCVs using antibodies against MTV1 and NEV/AGD5. In both cases, CCVs were clearly labeled by the antibodies (Figures 7E and 7F, arrowheads), which indicates that MTV1 and NEV/AGD5 are present on CCVs and thus probably participate in their formation at the TGN. Labeling was found only in part of the vesicles, which may represent the TGN-derived CCVs, while the unlabeled vesicles may represent the plasma membrane–derived CCVs.

It follows that CCV formation may be disrupted in the *mtv1 nev/agd5* double mutant, and this could potentially lead to alterations in morphology or size of the TGN. To test this hypothesis, we analyzed the ultrastructure of root meristem cells of the *mtv1 nev/agd5* double mutant by electron microscopy. We repeatedly observed morphological alterations at the trans-side of the Golgi and the TGN, with grossly curved cisternae that gave the TGN a bloated appearance (Figures 8A and 8B). This is very similar to what has been reported in the cells of *nev/agd5* mutant flowers (Liljegren et al., 2009) or for multiple mutants in the TGN-localized SNAREs of the SYF4 group (Uemura et al., 2012). By contrast, we did not observe clear changes of the PVC, although we cannot exclude subtle morphological changes or alterations in size or number, which would only be detectable by a quantitative analysis of a large number of electron microscopy images. The changes in morphology support that MTV1 and NEV/AGD5 participate in CCV formation at the TGN. To further substantiate this hypothesis, we analyzed the subcellular distribution of VSRs, which are potentially incorporated into CCVs at the TGN (Hinz et al., 2007). We found that VSRs copurified with CLC, MTV1, and NEV/AGD5 in the isolated *Arabidopsis* CCV fractions (Figure 7D), consistent with prior evidence of VSR association with CCVs in *Pisum sativum* cotyledons (Kirsch et al., 1994; Hinz et al., 1999). We fractionated microsomes in Suc density gradients and compared the VSR profiles in samples from wild-type and mutant plants. In wild-type plants, VSRs were broadly distributed in the gradient (Figure 8C), consistent with a steady state localization primarily at the TGN (lighter fractions) and the PVC (denser fractions) (Rojo et al., 2003b). The distribution of VSRs was not significantly changed in *mtv1* plants but was weakly altered in *nev/agd5* plants and very strongly affected in *mtv1 nev/agd5* plants, being displaced toward the bottom of the gradients. A shift to denser fractions was also observed for the PVC syntaxin SYP21 fractions in the *mtv1 nev/agd5* mutant (Figure 8C), albeit to a lesser degree. The drastic change in VSR gradient distribution is consistent with defects in CCV-mediated transport of VSRs from the TGN to the PVC. Interestingly, the shift in VSRs distribution was accompanied by a strong increase in VSR abundance in *mtv1 nev/agd5* plants (Figures 8C and 8D), a response that is common to other mutants with a disrupted VSR trafficking cycle (Yamazaki et al., 2008; Zhour et al., 2009; Uemura et al., 2012). Taken together, the subcellular localization at the TGN and in CCVs, the defects in VSR and ALEURIN distribution, and the observed morphological alterations of the TGN strongly suggest a role for MTV1 and NEV/AGD5 in CCV formation at the TGN.

**MTV1 and AGD5 Physically Interact with Clathrin**

Several ENTH domain proteins, including EPSIN1, EPSINR2, and AtECA1 in *Arabidopsis*, contain specific motifs for direct binding to clathrin (Song et al., 2006a; Lee et al., 2007; Song et al., 2012). In addition, two ARF GAPs from mammals, SMAP1 and 2, have also been shown to interact physically with clathrin (Tanabe et al., 2005; Natsume et al., 2006). To elucidate whether NEV/AGD5 and MTV1 may interact with clathrin, we first searched for putative clathrin binding motifs in the primary amino acid sequence. Neither has a prototypical LxPxL (L = Leu, P = polar; x = bulky hydrophobic residue) clathrin binding box (Lafer, 2002), but MTV1 contains a slightly modified version (LIDTGD) that conforms to the clathrin binding motifs from the mammalian proteins GGA3, ACK1, and EPSIN1 (Dell’Angelica et al., 2000). Moreover, this motif is highly conserved among plant orthologs of MTV1 (see Supplemental Figure 9 online), suggesting that it is functionally relevant. In addition, MTV1 has five DLL or DxW-related motifs situated in the C-terminal half of the protein (Figure 9A; see Supplemental Figure 9 online), which were shown to be required for the clathrin binding activity of the human ENTH domain–containing protein Auxillin (Scheele et al., 2003) and are also found in other epsins.

To test whether MTV1 interacts with clathrin and to characterize in that case where the binding motifs reside, we performed pull-down experiments using three glutathione S-transferase (GST)–fused constructs of MTV1: the full-length protein, the N-terminal part (amino acids 1 to 134) containing the ENTH domain, and the rest of the protein, which contains the putative clathrin binding motifs. The pull-down assays showed clear binding of CHC to full-length MTV1 and to the C-terminal construct, but not to the N-terminal ENTH domain (Figures 9B and 9C). These results indicate...
that MTV1 directly binds clathrin, most likely through the clathrin binding motifs present in the C-terminal half of the protein, similar to what has been shown in canonical Epsins.

As clathrin binding motifs were not detected in the primary sequence of NEV/AGD5, we used tandem affinity purification to identify in vivo partners of NEV/AGD5. TAP-AGD5 was stably transformed in Arabidopsis cell cultures, and copurified proteins were identified by mass spectrometry. We performed two independent experiments and in both of them we could identify with very high confidence CHC copurifying with the bait (Figure 9D; see Supplemental Table 2 online). Interestingly, when the tag was fused to the C terminus of NEV/AGD5 (AGD5-TAP), clathrin interaction could not be identified in two independent experiments, which might indicate that the tag at this position masks important domains required for the interaction. Taken together, these experiments indicate that NEV/AGD5 and clathrin are closely associated in vivo, most likely through direct protein–protein interaction.

The interaction with clathrin fully supports the genetic and cell biological evidence, suggesting that MTV1 and NEV/AGD5 function in CCV formation at the TGN for transport of vacuolar cargo to the PVC.

DISCUSSION

The Molecular Mode of Action of MTV1 and NEV/AGD5

Our results show that the biological function of both MTV1 and NEV/AGD5 must lie in an anterograde, clathrin-dependent trafficking pathway from the TGN to the vacuole. The most likely
Figure 7. MTV1 and NEV/AGD5 Are Associated with CCVs.

(A) Live-cell imaging of root epidermal cells harboring MTV1-mCherry (in red) and CLC-GFP reveals partial overlap in the cell interior but not at the plasma membrane.

(B) After 1 h treatment with 50 µM BFA, MTV1-Cherry and CLC-GFP localize to the periphery of the BFA core.

(C) Purification of CCVs from Arabidopsis T87 cell cultures and electron microscopy image of purified fraction.

(D) Immunoblot analysis of enriched CCV fractions; 3 µg of protein was loaded in each lane. 1, Cell homogenate; 2, 30,000g supernatant; 3, Suc step gradient load; 4, linear D2O/Ficoll gradient load; 5, final enriched CCV fraction. Antibodies used: CHC, AP1γ-subunit (γ AP1), MTV1, NEV/AGD5, VSR1, SEC12 (endoplasmic reticulum), and RGP1 (Golgi/cytosolic).

(E) and (F) Immunogold labeling of embedded CCVs using anti-MTV1 (E) or anti-NEV/AGD5 (F) antibodies visualized with gold nanoparticle-conjugated secondary antibodies. Arrowheads point to CCVs with immunogold signal; inset is an enlargement of the region outlined by the white box. Bars = 500 nm.
molecular mode of action is a role in the productive formation of cargo-filled CCVs at the TGN. For MTV1, the literature about other ENTH domain–containing proteins, namely, epsins, supports this notion. Epsins have been shown to recognize and bind certain phosphoinositides in target membranes by their bulky ENTH domain. This domain partially enters into the lipid bilayer and dislodges the head groups, thereby introducing membrane curvature toward the cytosolic face or at least reducing the energy required for membrane bulging. Their long, disordered C-terminal part then serves as an adaptor for clathrin and adaptor complex subunits. Thus, epsins and at least part of other ENTH domain–containing proteins can be seen as adaptors involved in CCV formation. In the case of MTV1, our characterization strongly suggests such an adaptor role for CCV formation at the TGN.

For ARF GAPs, the current understanding of their molecular mode of action is not as undisputed. Most studies have centered on the prototypical mammalian ArfGAP1, which is involved in COPI vesicle formation. Knowledge about ARF GAPs involved in CCV formation is much more limited, although the basic mechanisms may well be the same (reviewed in Kon et al., 2011), and a recent study found an unexpected involvement of ArfGAP1 also in CCV formation (Bai et al., 2011). The initial hypothesis that ArfGAP1 triggers vesicle coat disassembly by the ARF-GTP to ARF-GDP conversion has been revisited over the last 10 years, and several, more refined models for ARF GAP function have been proposed (reviewed in Nie and Randazzo, 2006; Spang, et al., 2010).

All agree that GTP-bound ARFs recruit coat proteins and that ARF GAP–dependent GTP-GDP hydrolysis results in coat disassembly. The debate centers on the question of how the GTPase activity of ARFs is regulated, as early activity would result in premature coat dissociation and nonproductive vesicle formation. A recent model proposes a dual function for ARF GAP: (1) It acts in cargo recruitment at the membrane and facilitates interaction with GTP-bound ARF for coat assembly. (2) Once the coat is assembled and the vesicle has budded, ARF GAP activity commences, leading to GTP hydrolysis and coat disassembly (Spang et al., 2010). This implies that ARF GAPs act in cargo sorting and are part of the coat itself, which is in line with our observation that NEV/AGD5 is present on CCVs.

**Figure 8.** The mtv1 nev/agd5 Double Mutant Has Altered Post-Golgi Properties.

(A) and (B) Ultrastructure of Golgi apparatus and TGN in Col-0 (A) and mtv1 agd5 (B) root epidermal cells. Frequently, the trans-facing Golgi cisternae were bent into sickle shaped or fully circular structures. Bars = 200 nm.

(C) Suc density gradient of total (S1) protein extract of Col-0, mtv1, agd5, and mtv1 agd5, probing with VSR or SYP21 antiserum.

(D) Quantitative immunoblot comparing total protein extracts of Col-0, mtv1, nev/agd5, and mtv1 nev/agd5. Ponceau staining as loading control.
Arabidopsis NEV/AGD5 has been previously reported as a TGN-localized AGD that interacts with Arf1 (Liljegren et al., 2009; Stefano et al., 2010). Interestingly, these studies showed that NEV/AGD5 shares substantial sequence similarity in the ARF GAP domain to the mammalian Arf GAPs SMAP1 and SMAP2, both of which directly interact with CHC (Tanabe et al., 2005; Natsume et al., 2006). Our results suggest that NEV/AGD5 also interacts directly with CHC with an uncharacterized binding domain. The localization and interaction data of NEV/AGD5 and Arf1 as well as multiple, additional reports of Arf1 localization at the TGN (Tanaka et al., 2009; Stierhof and El Kasmi, 2010; reviewed in Robinson et al., 2011), add further weight to the hypothesis that Arf1 is involved in CCV formation at the TGN (Pimpl et al., 2003), in addition to its well established function in COPI vesicle formation at the Golgi (Lee et al., 2002; Takeuchi et al., 2002; Stefano et al., 2006). A recent report shows that Arf1 is recruited to the Golgi by the action of AGD8 and AGD9 (Min et al., 2013), so it may be possible that NEV/AGD5 recruits Arf1 to the TGN. Further experiments will be needed to clarify this question. Also, it needs to be determined whether NEV/AGD5 interacts with other ARFs in vivo, following up on the result that NEV/AGD5 in vitro can interact promiscuously with the plasma membrane–localized ARFB (Stefano et al., 2006).

Functional Redundancy for MTV1 and NEV/AGD5

Despite their presumed central roles in clathrin-dependent trafficking from the TGN to the vacuole, the single mtv1 and nev/agd5 mutants display relatively mild defects in vacuolar trafficking and no obvious overall growth or developmental aberrations. The most likely explanation for the absence of more severe phenotypes is functional redundancy with other members of the respective protein families. Based on sequence similarity and domain structure, NEV/AGD5 is a class II AGD and groups together with AGD6-AGD10 (Vernoud et al., 2003). Phenotypes that reveal their biological function might only manifest in multiple loss-of-function mutants, as is the case for AGD8 and AGD9 (Min et al., 2013), or in tissues where a single AGD is the only or the dominant AGD expressed, as for example RPA/AGD10 in root hairs (Song et al., 2006b). This is likely the case for NEV/AGD5 in the floral abscission zone, where the expression pattern and the trafficking defects of the single nev/agd5 mutant (Liljegren et al., 2009) suggest that NEV/AGD5 is the dominant AGD. In other plant tissues, functionally redundant AGDs may largely compensate for the loss of NEV/AGD5.

For MTV1, the situation is more complex, as the 43 ENTH domain–containing proteins in Arabidopsis show (with few exceptions) little sequence similarity among each other outside their ENTH domain, which makes predictions about their functional similarity difficult. So far, all other ENTH proteins analyzed (Epsin1, Epsin2R, ECA1, ECA2, and ECA4) interact with clathrin, even though they lack a canonical clathrin binding box. However, there is only limited data about their biological function, and the only known mutant with a vacuolar transport defect is epsin1 (Song et al., 2006a). This mutant has, like mtv1, no obvious overall phenotype (Song et al., 2006a), and so it is likely that also in this case other ENTH domain–containing proteins compensate for its lack. For Epsin2/2R, no direct functional data...
are available (Lee et al., 2007), whereas ECA1, 2, and 4 localize to the plasma membrane and cell plate (Song et al., 2012), making a direct involvement in vacuolar trafficking unlikely.

Contrary to the single mutants, the mtv1 nev/agd5 double mutant shows pronounced vacuolar trafficking defects and is severely affected in overall growth. Nevertheless, vacuolar trafficking is by no means completely abolished. For instance, mtv1 nev/agd5 seeds contain high levels of mature seed storage proteins. This can be explained in two ways, which are not mutually exclusive: (1) The abovementioned redundancy for both MTV1 and NEV/AGDS with proteins of similar function, which may be more prevalent in certain tissues, such as in seeds, and (2) the presence of alternative machinery and pathways for TGN to PVC transport of vacuolar cargo. For example, in seeds, dense vesicles are abundant and may compensate for the reduced CCV-dependent transport of vacuolar cargo in mtv1 nev/agd5 mutants, which would also explain the relatively weak developmental phenotypes observed in these tissues.

**METHODS**

**Plant Growth Conditions**

Plants were grown on soil in the greenhouse or walk-in chambers at 22°C under a 16/8-h light/dark regime. For axenic culture, seeds were grown on vertical plates at 22°C in a 16/8-h light/dark cycle on 0.5× Murashige and Skoog (MS) medium with 1% Suc and 0.4% phytagel. For horizontal growth, phytagel was substituted for 0.8% agar.

**Mutants and Transgenic Lines**

mtv1-1 and mtv1-2 were isolated from an EMS-mutagenized population derived from the L1 transgenic line (Sanmartín et al., 2007). This population was previously described (Sohn et al., 2007). The mutant loci were identified by bulk segregant analysis and subsequent fine mapping using appropriate indeel and cleaved-amplified polymorphic sequence markers. The mtv4-2 (Salk_079928; identical to nev-7 in Liljegren et al., 2009) and mtv1-2 (Salk_061811) SALK T-DNA lines were used for functional analyses, unless otherwise noted. The following subcellular marker lines have been described previously: wave2R (=mCherry-RabF2b/ARA7), wave22R (=mCherry-STLP2), wave129R (=mCherry-RabA1g) (Geldner et al., 2009); SYLP1-CFP (Robert et al., 2008); Va1-RFP (Dettmer et al., 2006); pPIN1-PIN1-GFP (Bencoková et al., 2003); pPIN2-PIN2-GFP (Abas et al., 2006); SP-RFP (Hunter et al., 2003); PIN2-GFP (Abas et al., 2006); SP-RFP (Hunter et al., 2003); and CLC-GFP (Konopka et al., 2008), MTV1 and NEV/AGDSfusions to GFP, GUS, mRFP, mCherry, and TAP-Tag were realized using Gateway technology and the primers indicated in Supplemental Table 3 online for cloning into pDONR207 or pDONR221 entry vectors. An ∼500-bp promoter region upstream of ATG was used for pMTV1-MTV1-GUS and pMTV1-MTV1-GFP, and ∼1.5 kb was used for pAGDS-AGDS-GUS. Gateway subcloning into binary destination vectors pGW804 and pGW803 (Nakagawa et al., 2007) and pUbI N-RFP (Greven et al., 2010) and Agrobacterium tumefaciens-mediated transformation was used to generate the stable transgenic plants or cell cultures indicated in the text. At least five independent transformants were analyzed for each stably transformed line.

**Phylogenetic Analyses**

Phylogenetic analyses were performed in Jalview (Waterhouse et al., 2009) using the Muscle alignment algorithm with default parameters. Neighbor joining was used to calculate the phylogenetic tree, and bootstrap values for 1000 iterations are indicated.

**Protein Detection and Antibodies**

The following previously described primary antibodies were used in immunoblots at 1:1000 concentration unless indicated otherwise: anti-NEV/AGDS (Liljegren et al., 2009), 1:5000; anti-ALEURAIN (Ahmed et al., 2000); anti-CPY (Rojo et al., 2003a); anti-CHC (BD Biosciences 610499); anti-GST (Carl Roth 3998), 1:2000; anti-SYP21 (Da Silva Conceição et al., 1997); anti-VSR (Ahmed et al., 2000); anti-vAP-1 (kindly provided by I. Hwang); anti-SEC12 (Bar-Peled and Raikhel, 1997); anti-RGPI (Delgado et al., 1998), anti-BIP (Santa Cruz Biotechnology sc-33577), antiactin (Sigma-Aldrich A04080), 1:2000; and anti-DT3 (kindly provided by K. Schumacher; Schumacher et al., 1999), 1:2000. Anti-MTV1 antisera was raised in rabbit against an MBP-MTV1 full-length fusion protein. Secondary antibodies were horseradish peroxidase–conjugated anti-mouse (NA931V; GE Healthcare) and anti-rabbit (NA934V) at 1:10,000. Seed proteins were extracted and analyzed as described (Zouhar et al., 2010). Isolation and analysis of extracellular fluid were performed as described (Sanmartín et al., 2007). Density gradient fractionation was performed as described (Bassham and Raikhel, 1998). All immunoblot experiments were repeated at least three times.

**CCV Isolation and Microscopy**

T87W cells (60 to 90 mL packed cell volume) were lysed by nitrogen decompression, and CCVs were purified by differential and density gradient centrifugation, essentially as described (Depta and Robinson, 1998; Harley and Bevers, 1989). In brief, the cell homogenate (H) was subjected to sequential differential centrifugation at 100g, 1000g, and 30,000g. Microsomal membranes (SGL) from the 30,000g supernatant (S30) were concentrated by sedimentation (120,000g) and layered onto a Suc step gradient. The Suc step gradient was centrifuged (116,000g), and fractions corresponding to the 10 and 10/40% (v/v) Suc steps were harvested, from which membranes were concentrated by sedimentation (180,000g). The resulting pellet was resuspended (DFGL), layered over a linear D20/Ficoll gradient, and centrifuged (80,000g). D20/Ficoll gradient fractions containing enriched CCVs (equivalent to ∼14 to 16% [v/w] Suc) were harvested and concentrated by sedimentation (284,000g) to yield the final enriched CCV fraction, typically 100 to 300 µg. For immunoblot analysis, aliquots of subcellular fractions (H, S30, SG-load, DGF-load, LSG-load, and CCV) were solubilized in 1 × Laemmli loading buffer. Protein concentration was quantified using Pierce 660 protein assay reagent containing ionic detergent compatibility reagent using BSA as a standard according to the manufacturer’s instructions (Thermo Fisher Scientific). For negative stain electron microscopy analysis, enriched CCV samples were adjusted to 2% (w/v) aqueous osmium tetroxide, dried as a thin layer onto the surface of a pioloform-coated 200 mesh nickel grid, and stained with Nano-W methylamine tungstate (catalog number 2018; Nanoprobes). Imaging was performed on a Philips CM120 transmission electron microscope (Philips/FEI) with a MegaView III side-mounted digital camera at the UW-Madison Medical School Electron Microscope Facility. Quantitative analysis of CCVs versus uncoated vesicles was performed on randomly selected electron microscopy images of negative-stained samples using the ImageJ (http://rsb.info.nih.gov/ij/) Cell Counter plug-in. Coated vesicles represented, on average, 76% (n = 11) of the total vesicles counted.

**MTV1 Pulldown**

The full-length coding sequence of MTV1, a region encompassing the ENTH domain (amino acids 1 to 134), or the C terminus (amino acids 135 to 699) was cloned into pDONR221 using primers indicated in Supplemental Table 3 online and subcloned into a Gateway modified version of pGEX-2T (GE Healthcare) to yield GST-MTV1, GST-ENTH, and GST-C-Term. Proteins were expressed in Escherichia coli BL21 cells, extracted in lysis buffer
The control of the constitutive capping activity of KNTAP and KCTAP to yield TAPtag-AGD5 and AGD5-TAPtag under the control of the constitutive cauliflower mosaic virus 35S promoter (Van Leene et al., 2007). Constructs were transformed into Arabidopsis thaliana cell suspension cultures by Agrobacterium co-cultivation (Van Leene et al., 2007). Tandem affinity purification of protein complexes was done using the GS tag (Van Leene et al., 2008), as described by Van Leene et al. (2011). Mass spectra were acquired using a 4800 Proteomics Analyzer (Applied Biosystems), and MS-based protein homology identification was based on The Arabidopsis Information Resource genomic database (Van Leene et al., 2010). Experimental background proteins were subtracted based on ~40 TAP experiments on wild-type cultures and cultures expressing TAP-tagged mock proteins GUS, RFP, and GFP (Van Leene et al., 2010).

**Ultrastructural Analysis and Immunogold Labeling**

Ultrastructural analysis of high-pressure frozen, freeze-substituted (osmium tetroxide in acetonitrile and epoxy resin-embedded root tips was performed as previously described (Reichert et al., 2007).

For immunolabeling, roots from 10-d-old wild-type and MTV1-GFP Arabidopsis seedlings were high-pressure frozen/freeze-substituted in a Baltec HPM010. Samples were then substituted in 0.2% uranyl acetate (Electron Microscopy Sciences) plus 0.2% glutaraldehyde (Electron Microscopy Sciences) for 72 h and warmed to 50°C for 24 h. After several acetone rinses, these samples were infiltrated with Lowicryl HM20 (Electron Microscopy Sciences) for 72 h and polymerized at ~50°C under UV light for 48 h. Sections were mounted on Formvar-coated nickel grids and blocked for 20 min with a 5% (w/v) solution of nonfat milk in Tris-buffered saline containing 0.1% Tween 20. The sections were incubated in the primary polyclonal antibodies against anti-GFP (Torrey Pines BioLabs) for 1 h, rinsed in Tris-buffered saline containing 0.5% Tween 20, and then transferred to the secondary antibody fragment (Fab'2-goat anti-rabbit 1:10; Electron Microscopy Sciences) conjugated to 15-nm gold particles for 1 h. Labeling quantification was performed using Fiji (http://fiji.sc/Fiji).

**Chemical Treatments**

At 4 to 5 DAG, vertically grown seedlings were incubated in 1 mL liquid 0.5 MS medium with 1% Suc in 24 microwell plates with gentle agitation. Drug concentrations used were 50 μM BFA for durations between 30 min and 2 h and 30 μM wortmannin for 1 h. FM4-64 was used at 2 μM, either as a prestrain for 5 min on ice, followed by washing twice for 1 min in liquid medium on ice, or, for long-term treatments, remaining for 3 h in the incubation medium. All chemicals were dissolved in DMSO and controls carried equivalent volumes of DMSO.

**Wide-Field and Confocal Microscopy**

Alexander stain for pollen viability was performed as described (Peterson et al., 2010) and observed with a Leica DRE upright microscope with a ×20 0.75 × 20 0.75 apochromatic objective. Confocal imaging was performed on an inverted Leica SP5II, using a ×63 1.2 water immersion objective for subcellular features or a ×20 0.75 dry objective for overview images. The pinhole for colocalization experiments was adjusted to ensure confocality. Photomultiplier tube settings were chosen to avoid amplifier saturation, and line averaging was employed to avoid between-frame registration errors due to mobile particles. Colocalization and drug treatment experiments were performed at least three times, in each observing at least 10 individual roots.

**Accession Numbers**

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: MTV1, AT3G16270; MTV4/NEV/AGD5, AT5G54310.

**Supplemental Data**

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

- **Supplemental Figure 1.** T-DNA Insertion Mutants and the Original EMS Alleles of mtv1 and mtv4 Are Phenotypically Equivalent.
- **Supplemental Figure 2.** Phylogenetic Analysis of MTV1 and the Previously Characterized ENTH Domain–Containing Proteins Epsin1, Epsin2/2R, and Epsin3.
- **Supplemental Figure 3.** Seed Protein Profile and Analysis of Total Leaf Extracts.
- **Supplemental Figure 4.** Endocytic Uptake of FM4-64.
- **Supplemental Figure 5.** PIN1-GFP Polar Localization, Formation of BFA Bodies, and Washout in Col-0 and mtv1 nev/agd5 Double Mutant.
- **Supplemental Figure 6.** Colocalization of MTV1-GFP and Subcellular Markers in Cotyledon Pavement Cells.
- **Supplemental Figure 7.** First, Middle, and Last Frames of Supplemental Movies 1 and 2 Online.
- **Supplemental Figure 8.** Immunogold Labeling of MTV1-GFP.
- **Supplemental Figure 9.** Multiple Sequence Alignment of MTV1 Orthologs.
- **Supplemental Table 1.** Quantitative Analysis of Immunogold Labeling.
- **Supplemental Table 2.** Protein Identification Data of the NEV/AGD5 TAP-Tag Experiment.
- **Supplemental Table 3.** Primers Used for Cloning.
- **Supplemental Data Set 1.** Alignments Used to Generate the Phylogeny Presented in Figure 1D.
- **Supplemental Data Set 2.** Alignments Used to Generate the Phylogeny Presented in Supplemental Figure 2 Online.
- **Supplemental Movie 1.** Five-Minute Time-Lapse Video of MTV1-GFP (Green) and wave22R/SYP32-RFP (Red) in Root Epidermal and Cortical Cells.
- **Supplemental Movie 2.** Five-Minute Time-Lapse Video of MTV1-GFP (Green) and SYP61-CFP (Red) in Root Epidermal and Cortical Cells.

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MTV1 and MTV4 Encode Plant-Specific ENTH and ARF GAP Proteins That Mediate Clathrin-Dependent Trafficking of Vacuolar Cargo from the Trans-Golgi Network

Michael Sauer, M. Otilia Delgadillo, Jan Zouhar, Gregory D. Reynolds, Janice G. Pennington, Liwen Jiang, Sarah J. Liljegren, York-Dieter Stierhof, Geert De Jaeger, Marisa S. Otegui, Sebastian Y. Bednarek and Enrique Rojo

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