Possible titles:

An unexpected function for an ESCRT protein

Coordination of transport pathways to the plant vacuole

Multiple functions for the ESCRT protein ALIX

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The plant vacuole is a large multifunctional organelle that is critical for cell growth and turgor, ion homeostasis, protein storage, and degradation of macromolecules (1). These functions are maintained via several protein and membrane trafficking pathways to the vacuole, and defects in vacuolar trafficking and function often lead to severe growth and developmental phenotypes. Vesicle trafficking pathways that terminate at the vacuole include those delivering vacuolar proteins that are newly synthesized at the ER, plasma membrane or extracellular proteins that are endocytosed and delivered via multivesicular bodies (MVBs), and cytoplasmic components transported via autophagy (2, 3). The interconnections between these pathways, and how they are coordinately regulated to maintain appropriate vacuole size, protein content and function, remains unclear. In PNAS, Hu et al. (4) report an unexpected role in Arabidopsis for a protein, ALIX, previously shown to function in trafficking of plasma membrane proteins for degradation (5); that of delivery of newly synthesized proteins to the vacuole (Fig. 1).

Plasma membrane proteins destined for degradation in the vacuole are taken up by endocytosis and delivered initially to early endosomes, which in plants are equivalent to the *trans*-Golgi network (TGN), the site where biosynthetic and endocytic trafficking pathways meet (6). As the TGN matures into late endosomes/MVBs, vesicles bud inwards from the limiting membrane, pinching off to form luminal vesicles. This process requires the ESCRT (Endosomal Sorting Complex Required for Transport) machinery (7), which selects ubiquitinated membrane proteins for internalization, deforms the membrane, and pinches off vesicles into the lumen. In plants, detachment of vesicles is often incomplete, leading to a concatenated array of connected vesicles inside MVBs (8). MVBs then fuse with the vacuole, delivering their contents for degradation by vacuolar hydrolases. ALIX is a conserved ESCRT-associated protein that functions in MVB formation, and in Arabidopsis associates both with the ESCRT-III subcomplex, aiding cargo sorting and vesicle formation, and with the deubiquitinating enzyme AMSH, recruiting it to MVBs to recycle ubiquitin from cargo prior to degradation (5, 9). ALIX is therefore important for core ESCRT functions in trafficking of membrane proteins and MVB formation, in plants and in other eukaryotes.

Hu et al. (4) noticed that Arabidopsis *alix* mutants had more severe phenotypes in embryogenesis and seed germination than expected for defects in ESCRT function (10), leading them to look more closely for additional functions of ALIX. Arabidopsis and other plants contain two different types of vacuoles, depending on the developmental stage and cell type. Protein storage vacuoles (PSVs) are found

primarily in seeds, and are specialized for the accumulation of seed storage proteins, which are later degraded to provide resources for seed germination and early seedling growth. Lytic vacuoles (LVs), on the other hand, are acidic compartments specialized in the degradation of macromolecules, and are present throughout the plant. Proteins targeted to PSVs versus LVs have distinct sorting signals, and some differences in the trafficking machinery, although there is also significant overlap (11). Hu et al. discovered that ALIX is important for protein transport to both PSVs and LVs (4). A fluorescent marker for PSVs, transported to the vacuole in wild-type plants, was secreted from the cells of *alix* mutant embryos. Precursors of seed storage proteins, rather than the mature forms, accumulated in the *alix* mutants, and were found extracellularly instead of inside PSVs. LV markers were also secreted in mutant root cells, indicating that ALIX is required for efficient transport of proteins to both PSVs and LVs, and suggesting that it affects the function of sorting machinery common to both types of vacuoles.

To begin to assess the mechanism by which ALIX functions in the transport of vacuolar proteins, Hu et al. (4) identified ALIX-interacting proteins by co-immunoprecipitation from GFP-ALIX-expressing Arabidopsis plants, followed by LC-MS/MS. Major interacting factors were identified as VPS26A and VPS35A, two components of the core retromer complex. Retromer functions in the recycling of vacuolar sorting receptors (VSRs), a family of proteins that bind the sorting signals of PSV- and LV-targeted proteins to mediate their transport to the vacuole. Prior to reaching the vacuole, the VSRs release their cargo, allowing recycling of the VSRs by retromer and enabling their re-use in additional rounds of transport (12, 13). An interaction of ALIX with retromer therefore provides an intriguing hypothesis for why soluble vacuolar proteins are mis-sorted in *alix* mutants. ALIX was shown to be required for recruiting retromer subunits to membranes and for their stability, and therefore may have a key role in regulating retromer function. No interaction of retromer with other ESCRT components could be detected, suggesting that ALIX may have two distinct functions, interacting with ESCRT in membrane protein degradation and with retromer in soluble vacuolar protein transport. Support for this hypothesis was gained from the analysis of double mutants. Loss-of-function of both ALIX and retromer pathways was lethal, and partial loss-of function of both pathways enhanced defects in growth and in sorting to LVs and PSVs. These genetic interactions suggest that ALIX and retromer function together in trafficking to both types of vacuoles.

Hu et al. (4) hypothesized that the mis-sorting of LV and PSV proteins in *alix* mutants is due to defects in trafficking and localization of VSRs, given the function of retromer in VSR recycling. They assessed the subcellular localization of VSR1 by fluorescence and electron microscopy, and found that the VSR signal was punctate in WT cells, showing some co-localization with markers of MVBs, TGN and Golgi. In an *alix* mutant, decreased co-localization with MVB and TGN markers was seen, and instead VSR1 was found at the plasma membrane and on aggregated membrane clusters. This led to a model in which loss of ALIX caused decreased recruitment of retromer to membranes, leading to mis-localization of VSRs and in turn mis-sorting of vacuolar proteins.

This research also shed additional light on the long-controversial issue of the subcellular location at which VSR recycling occurs (14). The assumption that retromer recycles proteins from endosomes to the TGN, as occurs in animal cells (15), has been challenged by reports that retromer components localize to the TGN, that blocking retromer function causes accumulation of VSRs at the TGN (13), and that VSR recycles from the TGN to the Golgi, followed by reloading of ligands in the *cis*-Golgi (16). An alternative interpretation was reached by Kang et al. (17), whereby accumulation of soluble vacuolar cargo in a retromer knockdown mutant led to the proposal that VSR1 is recycled from MVBs to the TGN by

retromer. In the current paper, the authors performed a careful analysis of retromer subunit localization in both embryo and root cells, and showed that the majority localized to MVBs, where it overlapped with ALIX localization, rather than the TGN. Complicating these issues are the unexpected observations that in plants, the TGN is more acidic than MVBs (18), with implications for pH-dependent receptor-ligand binding, and that plants do not have a separate early endosome, with the TGN instead performing this function (6). This distinct organization of the endosomal system in plants, when compared to animals, means that observations in animal cells may not necessarily be valid in plant cells. Reconciliation of these disparate results will require additional research, and it is possible that retromer can recycle proteins from multiple endomembrane compartments. If the TGN matures into MVBs as has been suggested (19), then it is possible that a continuum of endosomal compartments exist from which retromer-mediated recycling may occur.

The factors that determine whether ALIX functions together with ESCRT or with retromer, and the mechanisms by which a single protein controls the activities of two different complexes and pathways, are open questions. It is possible that the balance between ALIX function in MVB formation or in VSR recycling is determined by the cell type or in response to environmental conditions. For example, degradation of plasma membrane proteins via the ESCRT pathway can increase in response to nutrient concentrations or environmental signals (9). The details of the regulation of these two distinct yet interconnected functions of ALIX remain to be elucidated.

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The author declares no competing interest.

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Figure legend

Fig. 1. ALIX functions in two distinct trafficking pathways. Left, ALIX interacts with retromer, mediating recycling of VSRs and therefore the sorting of soluble proteins to LVs and PSVs. Right, ALIX interacts with ESCRT, mediating the biogenesis of MVBs and degradation of ubiquitinated plasma membrane proteins.