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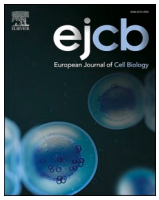

Retrograde transport in plants: Circular economy in the endomembrane system

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ABSTRACT

The study of endomembrane trafficking is crucial for understanding how cells and whole organisms function. Moreover, there is a special interest in investigating endomembrane trafficking in plants, given its role in transport and accumulation of seed storage proteins and in secretion of cell wall material, arguably the two most essential commodities obtained from crops. The mechanisms of anterograde transport in the biosynthetic and endocytic pathways of plants have been thoroughly discussed in recent reviews, but, comparatively, retrograde trafficking pathways have received less attention. Retrograde trafficking is essential to recover membranes, retrieve proteins that have escaped from their intended localization, maintain homeostasis in maturing compartments, and recycle trafficking machinery for its reuse in anterograde transport reactions. Here, we review the current understanding on retrograde trafficking pathways in the endomembrane system of plants, discussing their integration with anterograde transport routes, describing conserved and plant-specific retrieval mechanisms at play, highlighting contentious issues and identifying open questions for future research.

1. Introduction

Newly synthesized proteins that enter the endomembrane system can be retained at the endoplasmic reticulum (ER) or be transported in an anterograde direction until reaching another target compartment. In this anterograde direction, proteins may sequentially move through the Golgi apparatus, the *trans*-Golgi network (TGN) and the endosomes, finally reaching the plasma membrane (PM) and extracellular space or the vacuole/lysosome, a compartment that, among many roles, functions in protein turnover. Different routes mediate this anterograde transport in the biosynthetic pathway, in some cases bypassing some of the intermediate compartments (Aniento et al., 2022). Moreover, in the endocytic pathway, secreted proteins can be internalized and transported successively through early endosomes (EEs) and late endosomes (LEs) in their way to the vacuole/lysosome (Aniento et al., 2022). These anterograde trafficking routes are complemented by retrograde trafficking pathways that provide reversibility to the transport process. Retrograde trafficking has been reported from all post-ER compartments, including recent evidence for recycling from the yeast vacuole and the mammalian endolysosome (Hirst et al., 2018; Suzuki and Emr, 2018; Seaman, 2019). The main role of these retrograde pathways is to retrieve proteins and membranes for their reuse in earlier compartments, in a form of circular cell economy. Key protein cargoes of these retrograde recycling pathways are components of the trafficking machinery itself that move forward in the endomembrane system while exerting their activities. Peripheral trafficking factors are recruited transiently to membranes to perform their activities and they can then be released into the cytosol to participate in novel rounds of trafficking reactions. In contrast, integral membrane factors that function from the donor membrane and end up in the target membrane would need to be actively retrieved if they were to participate in novel rounds of trafficking. This includes two main types of trafficking factors, v-SNAREs, which are present in the membrane of the donor compartments and mediate fusion with t-SNAREs present in the target membrane, and sorting receptors, which recruit cargo at the donor compartment and target it to the next compartment. In addition, retrograde trafficking acts as a salvage system to recover proteins that have escaped from their intended localization. Examples of this are the retrieval of ER resident proteins from the Golgi through the H/KDEL-receptor, and the retrograde intra-Golgi transport of glycosylation enzymes to maintain their specific cisternal localization in the context of forward maturation of the cisternae. Here, we review current knowledge on pathways and

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mechanisms of retrograde trafficking from the different compartments of the plant endomembrane system, highlighting commonalities and differences with other eukaryotes. We discuss how retrograde retrieval balances anterograde transport to control the fate and subcellular distribution of proteins, with a particular emphasis on the recycling of vacuolar sorting receptors, plasma membrane proteins and SNAREs.

2. Retrograde transport in the early secretory pathway

The view on the ER-Golgi interface trafficking in plants is currently changing. It was demonstrated that certain components of the trafficking machinery responsible for anterograde (ER-to-Golgi) and retrograde (Golgi-to-ER) transport in plants co-localize, suggesting that Golgi stacks and ER-localized import/export domains move together, forming mobile secretory and recycling units (daSilva et al., 2004; Lerich et al., 2012). Based on analogous co-localization studies, similar bidirectional transport portals were suggested for *Pichia pastoris* and mammalian cells (Roy Chowdhury et al., 2020). Despite the fact that the role of COAT PROTEIN II (COPII) in anterograde transport from ER to Golgi (Phillipson et al., 2001) is currently challenged in both plant (McGinness et al., 2022) and animal systems (Westrate et al., 2020), the retrograde transport from *cis*-Golgi to the ER and the retrograde transport from *medial*- and *trans*-Golgi cisternae to *cis*-Golgi is unambiguously carried out by COAT PROTEIN I (COPI) vesicles with clear cargo specificity (Donohoe et al., 2007). Electron tomography revealed two types of COPI vesicles of the same diameter that differ in cargo staining, coat architecture and thickness, and spatial distribution along the Golgi stack. Vesicles that lack lumenal staining, designated COPIa, originate from the *cis*-Golgi cisternae, while vesicles with dense cores, named COPIb, bud from *medial*- and *trans*-Golgi cisternae (Donohoe et al., 2007, 2013).

Maintenance of the ER homeostasis strongly depends on the retrieval of the ER resident proteins that were transported to Golgi in the bulk-flow manner. ER lumenal proteins contain HDEL/KDEL motifs at the C-terminal that are recognized by the Erd2p receptor in yeast (Semenza et al., 1990) and its homologues in plants (Lee et al., 1993) and humans (Lewis and Pelham, 1990). In plants, this interaction likely takes place either in the pre-*cis*-cisternal compartment (Ito et al., 2018) or in the *cis*-most Golgi cisterna. Importantly, the lower pH in these compartments allows for binding of the KDEL/HDEL motifs to the ERD2 receptors. Subsequent conformational change exposes the COPI binding motif, as was shown for the chicken ERD2 homolog (Bräuer et al., 2019). Both the COPII binding motif, necessary for the anterograde transport, and the COPI motif, necessary for the retrograde transport, are highly conserved in the Arabidopsis homologues (Robinson and Aniento, 2020). It was also suggested that ERD2 may be actively promoting its integration into the COPI vesicles via interaction with the p24 proteins that continuously cycle between the ER and the *cis*-Golgi (Montesinos et al., 2014). A quadruple *p24δ3/δ4/δ5/δ6* loss-of-function mutant shows alterations in Golgi structure, accumulation of the ERD2 receptor in the *cis*-Golgi and secretion of ER-resident proteins (Pastor-Cantizano et al., 2018). In agreement with the observed phenotypes in plants, presence of both p24 proteins and KDEL/HDEL receptors was recently confirmed by proteomics in the COPI vesicles isolated from various mammalian cell types (Adolf et al., 2019). The Dsl1 tethering complex that captures Golgi-derived COPIa vesicles is conserved across all eukaryotic kingdoms (Kraynack et al., 2005; Tagaya et al., 2014). In Arabidopsis, a gene encoding an ortholog of the Tip20p subunit of the yeast Dsl1 complex was identified as *MAIGO2* (*MAG2*) in a genetic screen for missorting of endogenous vacuolar cargo (Li et al., 2006). MAG2 physically interacts with Qa-SNARE SYP81 and Qb-SNARE SEC20, ER-membrane localized SNARE proteins (Li et al., 2006), while SYP81 colocalizes with Qc-SNARE SYP72 in punctate structures (Lerich et al., 2012). Subsequent analyses identified three MAG2-interacting proteins (MIPs). MIP1 and MIP2 are homologous to the Dsl1p/ZW10 and Sec39p/NAG subunits of the yeast and mammalian Dsl1 complexes, while MIP3, a Sec1/Munc18 protein, is a plant-specific subunit (Li et al., 2013). In *Saccharomyces cerevisiae*, the Dsl1 complex was found uniformly distributed over the ER. However, the COPI vesicles do not fuse randomly across the ER membrane but rather at punctate structures distinct from ER exit sites (Schröter et al., 2016). In *Pichia pastoris*, the Dsl1 complex forms partial rings around long-lived ER exit sites (Roy Chowdhury et al., 2020). In contrast, Arabidopsis DSL1 complex was found to be constitutively linked to Golgi stacks irrespective of their motile status. Therefore, the fusion sites of COPIa vesicles must be defined by other marker. Based on colocalization studies performed in transiently transformed tobacco leaf protoplasts, it was proposed that SYP72 punctae may represent predetermined docking sites for Golgi stacks (Lerich et al., 2012). However, the precise molecular function of SYP72 in membrane fusion of COPIa vesicles with the ER membrane has not yet been experimentally determined. Treatment with latrunculin B revealed that retrograde COPIa-mediated transport likely occurs only when Golgi stacks are temporarily stationary. When Golgi stacks are moving, the COPIa vesicles stay tethered to the DSL1 complex and accumulate in the ER-Golgi interface (Lerich et al., 2012). A scaffold of tethering factors, among others the golgin AtCASP (Renna et al., 2005; Osterrieder et al., 2017), surrounds the bidirectional transport portals and ensures that the newly formed vesicles do not escape to the cytoplasm.

While the *cis*-Golgi cisternae function as sites of *de novo* membrane assembly and ER-protein retrograde sorting, the *medial*- and *trans*-Golgi cisternae serve primarily the biosynthetic functions of plant Golgi. Importantly, the observed strict localization of Golgi enzymes to the particular cisternae requires active retrograde transport of enzymes via COPIb-type vesicles from the antecedent cisterna to the cisterna with newly established identity (Donohoe et al., 2007). In yeast, the conserved oligomeric Golgi (COG) complex is an octameric tethering complex involved in COPI-mediated intra-Golgi retrograde transport of Golgi residents to counteract their misplacement due to the cisternal maturation and provides a means for introducing quality control points and for regulating the rate of cisternal maturation. The Arabidopsis genome encodes all eight subunits of the putative COG complex as single copy genes (Vukašinović and Žárský, 2016). COG3 and COG8 play central role in the COG complex organization and the corresponding *cog3* and *cog8* mutants show a lack of clear *cis-trans* polarity and a reduced length of Golgi stacks (Tan et al., 2016). Similar changes in the length of Golgi stacks were observed in the *embryo yellow* (*eye*) mutant, in which the *COG7* gene is disrupted (Ishikawa et al., 2008), and, together with an absence of obvious *cis-trans* polarity, also in the *cog6* mutant (Rui et al., 2020). COG3 and COG8 localize to the periphery of Golgi stacks (Tan et al., 2016), supporting a conserved role of the COG complex in retrograde intra-Golgi trafficking, but COG2 was reported to colocalize with the EXO70A1 exocyst subunit (Oda et al., 2015), suggesting that the Arabidopsis COG complex functions may extend beyond its canonical roles. The COPI vesicles also function in the biogenesis of the *trans*-Golgi network. Electron tomography experiments demonstrated that the newly formed, still Golgi-associated TGN (Ga-TGN) is reduced by approximately one third by membrane recycling to the new *trans*-Golgi cisternae, a process mediated by COPIb vesicles (Kang et al., 2011).

In vitro budding of putative COPI vesicles was reconstituted from cauliflower membrane fractions (Pimpl et al., 2000). More recently, a method for in vitro reconstitution of COPII vesicles from Arabidopsis cell cultures has been reported (Li et al., 2021). Developing a system to reconstitute and/or isolate COPI vesicles from the model Arabidopsis system would be key to advance our understanding of retrograde transport in the early secretory pathway of plants.

3. Recycling of vacuolar sorting receptors from endosomes

Given the importance of vacuolar seed storage proteins for human and livestock nutrition, unravelling their transport has been an important area of research for plant cell biologists. Over the years, many

components of the cellular machinery necessary for transport of vacuolar proteins have been identified, and their characterization has unveiled the pathways and compartments involved. One of the principal actors for transport of soluble proteins, of the kind of vacuolar seed storage proteins, are sorting receptors, which recruit cargo proteins in the lumen of the endomembrane system and mediate their targeting to the vacuolar/lysosomal pathway by interacting through their cytosolic domains with trafficking factors. Sorting receptors for cargo destined to the vacuole/lysosome have been identified in all eukaryotic kingdoms, indicating that, in most organisms, targeting to those compartments is not a default process but rather requires active sorting processes at some step during the transport pathway. In yeast and metazoans, vacuolar/lysosomal sorting receptors recruit their cargo at the TGN and sort it into clathrin-coated vesicles (CCVs) for anterograde transport to the next endosomal compartment. Then, after cargo release, the receptors are recycled back to earlier compartments in a process mediated by the Retromer complex and/or sorting nexins (SNXs). In yeast, a pentameric complex formed by the Retromer complex trimer (Vps26p, Vps29p and Vps35p) and a dimer of SNX with BAR-domain (SNX-BAR) proteins (Vps5p and Vps17p) is responsible for recycling the vacuolar sorting receptor Vps10p. The Retromer trimer provides cargo selection activity through the interaction of Vps26p and Vps35p with the cytosolic tail of Vps10p, while the BAR-domains of SNXs provide the membrane bending activity required for tubule/vesicle formation (Tu and Seaman, 2021; Yong et al., 2022). In mammals, two different complexes have been proposed to recycle the cation-independent mannose-6-phosphate receptor (CI-MPR) that sorts lysosomal hydrolases (Chen et al., 2019; Tu and Seaman, 2021). A SNX-BAR dimer of SNX1/SNX2 and SNX5/SNX6, the mammalian homologues of Vps5p and Vps17p, has been shown to bind directly to CI-MPR and mediate Retromer-independent recycling (Kvainickas et al., 2017; Simonetti et al., 2017). In addition, an alternative pathway mediated by Retromer and SNX3 has been shown to contribute to CI-MPR recycling from endosomes (Cui et al., 2019b). In this latter case, the cargo is recognized through a binding pocket at the interface between VPS26 and SNX3 (Lucas et al., 2016), whilst membrane bending activity is thought to be accomplished through membrane remodelling by direct contact of VPS26 and SNX3 with membranes and membrane deformation by the rigid arches formed by Retromer (Kovtun et al., 2018; Leneva et al., 2021). In yeast and mammals, the Retromer subunit VPS29 interacts with the SNX-BARs, but an interaction between the two subcomplexes in plants has not been demonstrated. There is evidence for the function of both Retromer and SNX-BAR proteins in vacuolar trafficking but whether they function together or not in receptor recycling is yet unresolved (see below). In addition, there are SNXs without BAR domains encoded in plant genomes, but their function has yet to be determined (Heucken and Ivanov, 2018).

Four different families of vacuolar/lysosomal sorting receptor proteins have been identified in eukaryotes (de Marcos Lousa and Denecke, 2016) but land plant genomes only encode members of one of the families, the vacuolar sorting receptors (VSR). VSRs are type-I transmembrane proteins that link recognition of soluble cargo in the lumen of endomembrane compartments to cargo sorting and transport through the interaction of their C-terminal tails with cytosolic membrane trafficking factors. The intricacies of receptor–cargo interactions have been revealed in crystal structures of VSR lumenal domains in complex with cargo sorting signals (Luo et al., 2014; Tsao et al., 2022). Moreover, several studies have analysed the motifs in the cytosolic domain that determine their subcellular localization (de Marcos Lousa et al., 2012; Shimada et al., 2018). The cytosolic tail mediates interaction with adaptor complexes AP-1, AP-2 and AP-4 (Sanderfoot et al., 1998; Happel et al., 2004; Park et al., 2013; Gershlick et al., 2014; Fuji et al., 2016). A strictly conserved tyrosine motif is required for the interaction with adaptor complexes and for proper sorting of VSR-dependent cargo to the vacuole (Sanderfoot et al., 1998; Happel et al., 2004; Fuji et al., 2016). In addition, transient expression experiments in tobacco leaf cells and protoplasts suggest that a strictly conserved leucine in the cytosolic tail is required for VSR recycling from multivesicular bodies (MVBs) (daSilva et al., 2005; Foresti et al., 2010), the equivalent compartment to yeast and metazoan LEs. Competition assays using this leucine-mutant indicate that recycling of VSRs is necessary for maintaining full vacuolar transport capacity in plants (Foresti et al., 2010). These analyses have established the importance of the cytosolic tail in transport of VSRs, but it is a very contentious issue where and how retrograde trafficking of VSRs is achieved.

VSRs were first isolated from CCV-enriched fractions and shown to interact specifically with the TGN localized mammalian AP-1 adaptor complex (Kirsch et al., 1994; Paris et al., 1997; Sanderfoot et al., 1998). Therefore, by analogy to yeast and mammalian models upheld at the time, a "classical model" was proposed postulating that VSR-cargo complexes are loaded by AP-1 into CCVs at the TGN for anterograde transport to the MVB, where cargo release and recycling of VSRs would take place (Paul and Frigerio, 2007). In metazoans, the CI-MPR sorts cargo into CCVs for transport from the TGN to the EE, the compartment where the biosynthetic and endocytic pathways merge. The CI-MPR is then recycled before the EE matures into the LE and fuses with the lysosome for turnover of proteins (Braulke and Bonifacino, 2009). In contrast, uptake experiments of the endocytic tracer FM4–64 revealed that the TGN corresponds to the EE in plants (Dettmer et al., 2006). This led to the proposal of an alternative "maturation model" postulating that the plant TGN/EE matures into the MVB/LE, eliminating the need of an anterograde vesicle trafficking step between these two compartments. According to this model, VSRs would sort cargo in an earlier compartment of the biosynthetic pathway and would release cargo at the TGN, where retrograde recycling of VSRs would take place (Robinson and Pimpl, 2014). From the TGN the cargo would reach the vacuole by simply staying in the lumen during maturation of TGN into the MVB. Evidence in favour of both classical and maturation models has been presented, but which has more weight?

3.1. The case for the classical model

Perhaps the strongest evidence for the classical model is the sites where VSRs have been found in plant cells. By and large, most of the available data supports that, at steady state, VSRs are mainly distributed between the TGN and the MVB. Importantly, these data include immunolocalization of the endogenous VSRs (Ahmed et al., 1997; Paris et al., 1997; Sanderfoot et al., 1998; Li et al., 2002; Tse et al., 2004; Otegui et al., 2006; Niemes et al., 2010a; Viotti et al., 2010; Scheuring et al., 2011; Jia et al., 2013; Cui et al., 2014; Gao et al., 2014), the localization of a functional GFP-tagged VSR1 construct under the endogenous promoter (Hu et al., 2022), and the subcellular fractionation of endogenous proteins in purified compartments (Heard et al., 2015). This distribution of VSRs means that their sorting activity is most likely required for cargo delivery from the TGN to the MVB, implying that cargo is not just retained by default in the TGN as it matures into MVB. It suggests rather that VSRs sort cargo into vesicles for anterograde trafficking from the TGN to the MVB. Consistent with a vesicle-mediated step at this stage, VSRs interact with TGN-localized AP-1 and AP-4 complexes, which could be involved in budding vesicles loaded with VSR-cargo complexes for their anterograde transport from the TGN to the MVB. In support of this, a mutation in the conserved tyrosine motif required for interaction with adaptor complexes results in retention of a GFP-tagged VSR in the TGN and its depletion from the MVB (daSilva et al., 2006; Foresti et al., 2010). Moreover, mutants in AP-1 and AP-4, as well as mutants in their accessory epsin partners, display defects in delivery to the vacuole of VSR-dependent cargo (Song et al., 2006; Sauer et al., 2013; Wang et al., 2013; Fuji et al., 2016; Müdsam et al., 2018; Heinze et al., 2020). Intriguingly, the AP-1 and AP-4 complexes segregate to different domains of the TGN (Fuji et al., 2016; Heinze et al., 2020; Shimizu et al., 2021), which may reflect an involvement in different transport pathways. AP-1 is present at a TGN subdomain that

contains clathrin and the R-SNARE VAMP721, which mediates exocytic transport to the PM, whereas AP-4 is present at a subdomain containing the R-SNARE VAMP727, which mediates trafficking to the vacuole through the MVB (Shimizu et al., 2021). The segregation of clathrin away from the AP-4 subdomain is supported by the closer proximity of clathrin to EPSIN1, the accessory epsin to AP-1, than to MTV1, the accessory epsin to AP-4 (Heinze et al., 2020). The co-localization with VAMP727 makes AP-4 the most likely adaptor complex to participate in vesicle-mediated transport of VSR-cargo complexes from the TGN to the MVB, whereas AP-1 may function in protein secretion (Shimizu et al., 2021) or in CCV-mediated retrograde trafficking, which is currently the proposed function of AP-1 in yeast and mammals (Matsudaira et al., 2015; Day et al., 2018; Casler et al., 2019). Despite the segregation from clathrin-enriched TGN domains, AP-4 and MTV1 have been shown to bind clathrin (Sauer et al., 2013; Shimizu et al., 2021) and to associate with purified CCV fractions (Sauer et al., 2013; Dahhan et al., 2022), suggesting that clathrin does play some role in AP-4 and MTV1-mediated vesicle budding, but most probably not in forming standard CCVs (Shimizu and Uemura, 2022). Interestingly, in Arabidopsis embryo cells VSRs and their cargo are loaded into dense vesicles derived from the TGN or the trans-most Golgi cisternae that lack a distinct coat and are larger and clearly distinguishable from typical CCVs (Otegui et al., 2006).

Another key observation supporting the classical model is that the VSRs present in MVBs are mainly found on their limiting membrane (Tse et al., 2004; Otegui et al., 2006; Viotti et al., 2010; Jia et al., 2013; Cui et al., 2014; Gao et al., 2014), suggesting that they avoid internalization into intralumenal vesicles (ILVs) to allow for their recycling back from MVBs. Consistent with this notion, Retromer and SNX-BARs, have been found, albeit not exclusively, at the MVB in plants. Most data published, including immunolocalization of the endogenous subunits and proteomic analysis of isolated compartments, agrees on the localization of plant Retromer at the MVB (Oliviusson et al., 2006; Jaillais et al., 2007; Kleine-Vehn et al., 2008; Yamazaki et al., 2008; Zelazny et al., 2013; Heard et al., 2015; Munch et al., 2015; Rodriguez-Furlan et al., 2019a; Hu et al., 2022), although the VPS29 subunit has also been reported to associate with the TGN in tobacco and Arabidopsis (Niemes et al., 2010a). In contrast, the localization of SNX-BARs in plants is much more controversial. Some reports suggest that SNX-BARs localize mainly at the MVB (Jaillais et al., 2006; Pourcher et al., 2010; Bayle et al., 2011; Ivanov et al., 2014; Heard et al., 2015) whereas others suggest that they are present primarily at the TGN (Niemes et al., 2010a; Stierhof et al., 2013). Whatever the case, it is safe to assume that Retromer is localized at the MVB, where it could function in retrieval of VSRs present in the limiting membrane. Importantly, it has been reported that Arabidopsis VPS35 co-immunoprecipitates with VSRs (Oliviusson et al., 2006), providing direct evidence that Retromer is indeed involved in VSR recycling. Moreover, analysis of mutants in genes encoding subunits of the Retromer complex in Arabidopsis has revealed their role in transport of VSR-dependent cargoes to the vacuole, including seed storage proteins (Shimada et al., 2006; Yamazaki et al., 2008; Kang et al., 2012; Zelazny et al., 2013). Notably, in the *vps29* mutant the ratio of VSR1 found in the TGN versus the MVB is reduced, consistent with perturbed VSR recycling from the MVB to the TGN (Kang et al., 2012). Together these results provide robust genetic support for a role of the plant Retromer complex in vacuolar cargo delivery, and suggest that it does so by mediating VSR recycling from the MVB, as postulated in the classical model. In line with VSRs being retrieved from the MVB in plants, the Arabidopsis homologue of RME-8, a key factor for CI-MPR recycling in metazoans (Popoff et al., 2009; Norris and Grant, 2020), localizes to the MVB in Arabidopsis and is also required for vacuolar protein transport (Tamura et al., 2007; Silady et al., 2008; Delgadillo et al., 2020).

3.2. The case for the maturation model

A key prediction of the maturation model is that VSRs are recycled back from the TGN before it matures into the MVB. In line with this, some reports suggest that SNX-BARs, which participate in vacuolar trafficking of VSR-dependent cargo, associate with the TGN (Niemes et al., 2010a; Stierhof et al., 2013), although other reports situate them at the MVB (Jaillais et al., 2006; Pourcher et al., 2010; Bayle et al., 2011; Ivanov et al., 2014; Heard et al., 2015). Overexpression of SNX2b in Arabidopsis protoplasts causes partial endosomal retention of the chimeric vacuolar cargo NTPP-GFP (Phan et al., 2008), while a triple mutant in the three Arabidopsis *SNX-BAR* genes displays defects in trafficking of seed storage proteins, albeit weaker than those observed in Retromer mutants (Pourcher et al., 2010). However, in these two studies the effect of SNX-BAR disruption on VSR subcellular distribution was not analysed. In another work, it was shown that expression of dominant negative SNX-BAR constructs causes retention of a GFP-tagged VSR construct at the Golgi and TGN, but only partially affects delivery of VSR-dependent cargo into the vacuole (Niemes et al., 2010a), which suggests that transport of cargo from the TGN onward may be VSR-independent and could occur through maturation of TGN into MVBs. Moreover, accumulation of VSRs at the TGN could reflect a role of SNX-BARs in recycling them from that compartment. Intriguingly, stronger interference with SNX-BAR function leads to the ER retention of GFP-tagged VSR and its cargo (Niemes et al., 2010b). VSR accumulation at the ER may reflect secondary effects of SNX-BAR disruption on anterograde trafficking. However, these results do suggest that VSR-cargo interaction can already occur in the ER, as had been previously reported (Watanabe et al., 2004; daSilva et al., 2005). The findings on SNX-BARS are compatible with VSR cargo recruitment and sorting occurring in the early secretory pathway, at the ER and/or the Golgi, and recycling of VSRs occurring from the TGN, as postulated in the maturation model. The other important prediction of this model is that there is no anterograde vesicle-mediated trafficking between the TGN and the MVB, which in the classical model was initially proposed to occur via CCVs. The expression of a dominant negative clathrin heavy chain construct (clathrin-hub), which should interfere with CCV formation, had an inhibitory effect on endocytosis but not on vacuolar protein trafficking, suggesting that the latter does not involve a CCV-dependent transport (Scheuring et al., 2011). To note however, dense vesicles and/or AP-4/MTV1-derived vesicles, rather than CCVs, are now the primary candidates to mediate anterograde TGN to MVB trafficking, which would explain the lack of inhibition by clathrin-hub. To try to gain direct evidence of TGN to MVB maturation, electron microscopy analysis of Arabidopsis roots was employed. It was found that MVBs are often attached to tubular structures, suggestive of connections with the TGN, which was interpreted as evidence of TGN-based biogenesis of MVBs (Scheuring et al., 2011). Moreover, several components of the Endosomal Sorting Complex Required for Transport (ESCRT) machinery, which generate the ILVs that characterize MVBs, are recruited already at the TGN membrane, suggesting that they may remain associated during maturation of the TGN into ILV-containing MVBs (Scheuring et al., 2011). However, 3D electron tomography studies have not found evidence of direct connections between the TGN and MVBs in plants (Wanner et al., 2013; Cui et al., 2019a). Instead, the most convincing data favouring the maturation model has been provided in two elegant studies by Pimpl and collaborators. In the first work, compartment-specific sensors to measure VSR-cargo interaction were generated by using nanobodies to lockdown the VSR4 lumenal ligand binding domain in different compartments. Cargo retention and Förster resonance energy transfer from the GFP-labelled VSR sensor to the RFP-labelled vacuolar cargo revealed cargo-interaction with sensors located at the ER and Golgi, but not with sensors located at the TGN and the MVB, suggesting that the cargo is released already at the TGN and proceeds from there through a VSR-independent maturation process (Künzl et al., 2016). Moreover, a secreted RFP marker endocytosed by protoplasts from the culture media, was first internalized into the TGN/EE and then passed through the MVB and reached the vacuole, despite the lack of vacuolar sorting signals in the protein. Hence, it was

proposed that trafficking from the TGN on to the vacuole is through compartment maturation and does not require active sorting (Künzl et al., 2016). However, this latter assay was not quantitative and potential secretion of the endocytosed RFP was not measured, so accumulation in the vacuole may reflect partial leakage into the vacuolar pathway from excessive uptake of a secreted marker. In a second study also using nanobody-based tools in protoplasts, VSRs present at the TGN were labelled with GFP and then their trafficking was assayed. In this way, evidence was obtained that VSRs are recycled back from the TGN to the Golgi for cargo-reloading (Früholz et al., 2018).

It is clear then that there are sound results supporting particular features of both models, which should be integrated in a common scheme describing the trafficking of VSRs in plants.

3.3. Can the data be reconciled in a unified model?

At the time of the landmark finding that the plant TGN functions as an EE (Dettmer et al., 2006) it was considered to be a plant-specific characteristic, and by parallelism with EE to LE maturation in metazoans, it fuelled the search for evidence of TGN to MVB maturation in plants. However, it was recently discovered that the TGN is also the early destination for bulk endocytosis in budding yeast (Day et al., 2018), where it is well established that the GGA clathrin-adaptor proteins mediate vesicle transport of Vps10p and its cargo from the TGN to the MVB (Bonifacino, 2004; Paczkowski et al., 2015; Yanguas et al., 2019). Hence, vesicle mediated trafficking of VSR-cargo complexes from the TGN/EE to the MVB/LE and recycling of the receptors therefrom would not be a plant exception but rather it may constitute an ancestral pathway conserved in yeast and plants. In plants, MVBs undergo a Rab5 to Rab7 conversion (Cui et al., 2014; Ebine et al., 2014; Singh et al., 2014), whereas in metazoans this conversion marks the progression from EEs to LEs (Rink et al., 2005; Nordmann et al., 2010; Poteryaev et al., 2010). This implies that plant MVBs progress through different stages, possibly including a recycling phase. In this regard, a recycling-defective VSR construct has been reported to accumulate in a distinct late MVB, which is largely depleted of a wild type VSR construct (Foresti et al., 2010). This supports that membrane proteins, including VSRs, are retrieved from MVBs prior to their fusion with the vacuole. The localization of endogenous VSRs and Retromer at the limiting membrane of the MVB strongly favours the assumption of recycling from this compartment. Even if SNXs-BAR proteins were to localize at the TGN in plants, as suggested in some studies, Retromer does associate with the MVB and could mediate VSR-recycling in a SNX-BAR-independent manner, as reported in metazoans (Cui et al., 2019b). Moreover, plants encode other SNXs proteins without BAR domains (Heucken and Ivanov, 2018) that could cooperate with Retromer for cargo recognition and membrane bending, as has been shown with SNX3/Grd19p in mammals and yeast (Lucas et al., 2016; Kovtun et al., 2018; Cui et al., 2019b; Leneva et al., 2021). Alternatively, factors other than SNXs proteins can mediate membrane recruitment of Retromer. Remarkably, it was recently found that Arabidopsis ALIX/AtBRO1, in addition to its canonical role in the ESCRT pathway of MVB biogenesis (Cardona-López et al., 2015; Kalinowska et al., 2015; Shen et al., 2016), is also involved in recruitment of VPS26 and VPS29 to the MVB membrane through direct interaction with both subunits (Bassham, 2022; Hu et al., 2022). ALIX may then serve to bridge VPS26, which can directly interact with membranes (Kovtun et al., 2018), with the cytosolic assembled VPS29-VPS35 subcomplex (Zelazny et al., 2013), and, together with RABG3f (Zelazny et al., 2013; Rodriguez-Furlan et al., 2019a), promote the recruitment of the Retromer complex to the MVB. Importantly, *alix* mutants display severe alterations in the subcellular distribution of VSRs and defects in delivery of VSR-dependent cargo into the vacuole (Hu et al., 2022), supporting that ALIX participates in Retromer-mediated retrieval of VSRs for maintaining full vacuolar trafficking capacity in plants.

All considered, we deem most plausible that VSRs sort their cargo from a specialized subdomain or a subset of TGN cisternae for anterograde trafficking in AP-4/MTV1-derived vesicles, and are then recycled from the MVB in a retromer-dependent manner. To challenge or validate this model several key questions will have to be answered, namely, where VSRs sort and release their cargo and which is the target compartment for recycling of VSRs. Measuring compartment-specific receptor cargo interaction is a challenging problem that was smartly tackled by Pimpl and collaborators (Künzl et al., 2016; Früholz et al., 2018). A similar approach can be used to generate different TGN-localized VSR sensors and ligands to asses for interaction with independent genetic tools. Regarding the question of the target compartment for VSR-recycling, it has been reported that endocytosed FM4–64 labels sequentially the TGN, MVB and tonoplast but not the Golgi or the ER, even after 2 h of the initial uptake (Tse et al., 2004; Matheson et al., 2007; Toyooka et al., 2009; Zhang et al., 2011). The lack of labelling indicates that the rate of retrograde membrane recycling from the TGN or the MVB to the Golgi or ER is very slow at best, questioning whether such a flux would be sufficient for efficient recycling of VSRs, and suggesting that retrieval is instead directed to a post-Golgi compartment. To identify the target compartment for recycling, it may prove valuable to look at the machinery directing this step in other eukaryotic organisms. In this regard, plant genomes encode homologues of the GARP complex, which tethers incoming vesicles carrying vacuolar sorting receptors back from the endosome to the TGN in yeast and mammals. Moreover, Arabidopsis mutants in GARP complex subunits display defects in delivery of vacuolar cargo, consistent with their role in vacuolar trafficking (Pahari et al., 2014; Delgadillo et al., 2020). Interestingly, functional fluorescent protein fusions to the GARP subunits VPS51 and VPS54 are localized to an ER and microtubule associated compartment (EMAC) that is distinct from the Golgi but, intriguingly, also from TGN compartments labelled with fluorescent SYP61 and VTI12 markers (Delgadillo et al., 2020). However, there are distinct domains and/or types of TGN compartments in plants (Bassham et al., 2000; Staehelin and Kang, 2008; Kang et al., 2011; Uemura et al., 2014, 2019; Renna et al., 2018; Heinze et al., 2020; Shimizu et al., 2021), so it is possible that the EMAC corresponds to a particular subset of TGN cisternae not labelled by SYP61 and VTI12. Based on the presence of these different types of TGNs in plants, we suggest a scheme that can reconcile the two models proposed for VSR sorting and recycling in plants (Fig. 1). VSRs may interact with cargo in early TGN cisternae, possibly corresponding to the EMAC, and sort the cargo in AP-4/MTV1-derived vesicles for transport to a late SYP61- and VTI12-labeled TGN cisternae, where the cargo would be released. The ESCRT machinery would be recruited to these late TGN cisternae that would progressively mature into MVBs. VSR recycling would occur during this maturation, possibly via two independent pathways mediated by SNX-BARs and by Retromer-ALIX, respectively. This would emulate the situation in yeast, where there is sequential retrieval of proteins from early and late endosomes (Best et al., 2020). It will be very important to characterize the ultrastructure of EMACs and determine their relation with other compartments of the endomembrane system and to specific subsets of TGN, in particular to the TGN cisternae where AP-4/MTV1 reside.

4. Recycling of endocytosed plasma membrane proteins

Receptors and transporters localized on the PM are essential for plants to rapidly respond to extracellular stimuli, so plants need to tightly maintain their abundance and activity. Clathrin-mediated endocytosis (CME) and membrane microdomain-associated endocytosis (MME) are major routes that mediate the internalization of PM proteins in plants (Robinson, 2015; Lacy et al., 2018; Shen et al., 2022). Endocytic cargoes in plant cells are accurately recognized and selectively incorporated into CCVs through specific sorting signals including amino acid motifs and ubiquitination. (Paez Valencia et al., 2016; Dubeaux and Vert, 2017). The internalized cargo proteins can then be either delivered to the vacuole for degradation or recycled back to the

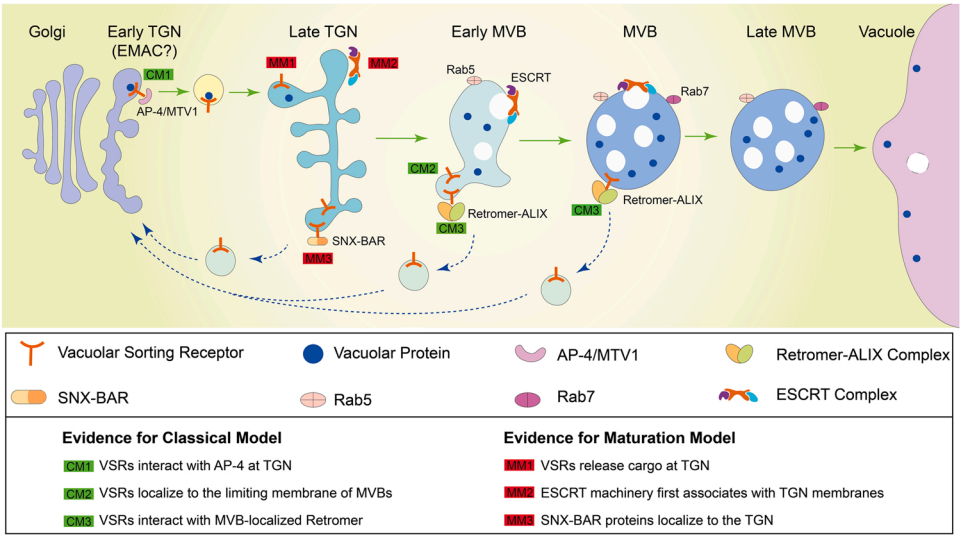

Fig. 1. Unified model for post-Golgi trafficking to the vacuole. VSRs recruit soluble vacuolar cargo in Early TGN cisternae and load them into vesicles through their direct interaction with AP-4 adaptor complexes. The AP-4/MTV1-derived vesicles fuse with a late TGN compartment, where VSRs release their cargo. ESCRT machinery associates with this late TGN cisternae, which gradually mature into early and late MVBs through internalization of proteins into ILVs and RAB5 to RAB7 conversion. VSRs are recycled from the late TGN via the SNX-BAR dimer. In the MVB, ALIX recruits Retromer to recycle the VSRs remaining in the limiting membrane. Finally, a late MVB devoid of VSRs can fuse with the vacuole and deliver the soluble cargo.

PM (Rodriguez-Furlan et al., 2019b). These two distinct fates depend on the sorting of the endocytosed proteins to different domains of the endosome, an ESCRT degradative-microdomain for internalization into ILVs and a SNX-Retromer recycling microdomain for retrieval of proteins to the PM (Norris and Grant, 2020). In *Caenorhabditis elegans*, SNX1 and its interacting partner RME-8 restrain the degradative microdomain through removal of the ESCRT-0 component HRS (Norris et al., 2017). Whether the plant homologues of SNX1 and RME-8 are also involved in maintaining a recycling microdomain in endosomes is yet unknown but their localization at the TGN/EE and MVB/LE is compatible with that idea, and should be tested in future research.

4.1. *ESCRT mediated sorting to the degradative pathway*

The conjugation of ubiquitin to PM proteins initiates endocytosis and ESCRT-mediated sorting into ILVs for degradation in the vacuole (Paez Valencia et al., 2016). The ubiquitin-mediated sorting is driven by ESCRT machinery, which consists of ESCRT-0, -I, -II, -III, and the Vps4/SKD1 (Vacuolar protein sorting 4 / SUPPRESSOR OF K+ TRANSPORT GROWTH DEFECT 1) subcomplexes (Paez Valencia et al., 2016; Gao et al., 2017). Mechanisms of ESCRT-dependent endosomal degradation have been summarized in recent reviews (Paez Valencia et al., 2016; Gao et al., 2017; Rodriguez-Furlan et al., 2019b). Basically, the ESCRT-0, a heterodimer of two ubiquitin-binding proteins, is responsible for capturing ubiquitinated cargoes and passing them to ESCRT-I subunits. ESCRT-I and ESCRT-II also contain ubiquitin-binding subunits, which deliver cargoes to downstream subcomplexes. ESCRT-III is recruited by ESCRT-II, driving the membrane invagination and ILV formation. Upon interaction with ESCRT-III subunits and binding to its activator Vps twenty associated 1 (Vta1), Vps4 assembles as hexamer on MVBs to promote ESCRT-III disassembly (McCullough et al., 2018).

Although ESCRT-0 is absent in plants, nine TARGET OF MYC PROTEIN1-LIKE proteins (TOLs) are encoded in Arabidopsis (Korbei et al., 2013). TOLs have been shown to bind ubiquitinated proteins and are required for endocytosis of PIN-FORMED (PIN)-type auxin carrier at the PM (Korbei et al., 2013). A recent study has shown that the Src homology-3 (SH3) domain-containing protein 2 (SH3P2) binds ubiquitinated proteins through its C-terminal SH3 domain (Nagel et al., 2017). SH3P2 interacts with the ESCRT-I component VPS23.1 and both VPS23.1 and SH3P2 co-localize in clathrin light chain (CLC)-positive punctae, indicating that they cooperate in internalization of ubiquitinated proteins in CCVs and their subsequent transfer to downstream ESCRT machinery. Indeed, VPS23.1 has been demonstrated to be involved in the ubiquitin-dependent degradation of the abscisic acid (ABA)-receptor in Arabidopsis (Yu et al., 2016). Remarkably, the deubiquitinating enzyme (DUB) AMSH3 can interact with both SH3P2 and VPS23.1 (Nagel et al., 2017). Thus, it is possible that SH3P2, VPS23.1, and AMSH3 can form a functional complex on CCVs in Arabidopsis. Importantly, by hydrolysing the ubiquitin chains, DUBs such as AMSH3 can rescue the cargo proteins from being sorted into the degradation pathway (Nagel et al., 2017). Arabidopsis DUB families include ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific proteases (UBPs) and ovarian tumor proteases (OTUs) (Isono and Nagel, 2014). So far, only AMSH3 (Isono et al., 2010; Nagel et al., 2017), UBP12, and UBP13 (An et al., 2018; Luo et al., 2022) have been shown to participate in the endosomal degradation of PM proteins. AMSH3 can interact with ESCRT-III and facilitate the hydrolysis of ubiquitin chains of cargos in Arabidopsis (Isono et al., 2010). UBP12 and UBP13 can recognize the brassinosteroid (BR) receptor BR insensitive1 (BRI1) on the PM (Luo et al., 2022). Moreover, UBP13 can bind and remove the polyubiquitin chains from BRI1, consequently delaying BRI1 degradation and positively regulating BR signaling. Additionally, overexpression of UBP13 inhibits the root meristem growth factor1 (RGF1) induced ubiquitination of its receptor RGFR1 (An et al., 2018). Recently, OTU11 and OTU12 were found to localize at the PM, probably through binding of their polybasic motifs to anionic lipids in the PM (Vogel et al., 2022). An important ensuing step will be to identify the complement of proteins deubiquitinated by the different membrane-associated DUBs and investigate how these fine-tune the ubiquitin-dependent degradation, localization and function of their targets.

4.2. *Recycling of endocytic cargo back to the PM*

In metazoans, two different mechanisms, involving Retromer and the related Retriever complex, have been shown to redirect deubiquitinated endocytosed proteins back to the PM (Chen et al., 2019). Retromer together with SNX27 and SNX-BAR proteins mediates recycling to the PM of cargo such as the glucose transporter GLUT1 (Simonetti et al., 2022). In addition, the Retriever complex together with SNX17 mediates recycling of distinct cargo to the PM, such as α5β1integrin (McNally et al., 2017). Plant genomes encode homologues of several components of the metazoan recycling machinery, including Retromer and Retriever subunits and SNX proteins. Metazoan Retriever is a heterotrimeric complex formed by the DSCR3/VPS26C, C16orf62/VPS35L and VPS29 subunits (McNally et al., 2017). The VPS29 subunit is common to Retromer and Retriever complexes, whereas the DSCR3/VPS26C and C16orf62/VPS35L subunits are distantly related, in terms of sequence similarity, with the Retromer VPS26 and VPS35 subunits, but have

conserved predicted structures (Chen et al., 2019). Plant genomes encode members of the distinctive DSCR3 clade of VPS26 family proteins, which in Arabidopsis is represented by VPS26C (Koumandou et al., 2011). Interestingly Arabidopsis VPS26C forms a complex with VPS35A and VPS29 that is distinct from the canonical Retromer complex, suggesting that plants do contain separate Retromer and Retriever complexes (Jha et al., 2018). Various reports link Retromer/Retriever and SNX-BAR complexes to recycling of proteins from endosomes in plants. For instance, *snx1* and *vps29* mutants have increased levels of PIN efflux carrier proteins in the vacuole and a concomitant reduction at the PM, consistent with a defect in rescuing PIN proteins from the vacuolar degradation pathway and recycling them to the PM (Jaillais et al., 2007; Kleine-Vehn et al., 2008). Moreover, *vps35a* plants show abnormal accumulation of various PM-proteins in an MVB-like endomembrane compartment, which implicates Retromer and/or Retriever complexes in recycling PM-proteins from endosomes (Nodzyński et al., 2013). SNX1 can be recruited by the microtubule-associated protein cytoplasmic linker-associated proteins (CLASPs) in a PX and BAR domain-dependent manner (Ambrose et al., 2013). CLASP binds microtubules, stabilizing and regulating SNX1 to redirect trafficking of PM proteins from the degradative vacuolar pathway to the recycling pathway towards the PM (Salanenka et al., 2018). *clasp-1* null mutants exhibit aberrant SNX1 endosomal association, suggesting the role of CLASP in SNX1 endosome localization. Treatment with the microtubule-depolymerizing drug oryzalin enhances PIN2 vacuolar degradation, further supporting the role of microtubules in PIN2 recycling to the PM (Hirano et al., 2015). Additionally, disruption of CLASP enhances PIN2 and BRI1 degradation in the vacuole, attenuating hormonal responses (Ambrose et al., 2013; Ruan et al., 2018). Therefore, CLASP maintains the homeostasis of auxin and brassinosteroid (BR) signaling by retrieving endocytosed receptors. The iron transporter IRT1 partially co-localizes with SNX1-GFP at the TGN and mutations in *SNX-BAR* genes cause increased degradation of IRT1 and reduced iron uptake, suggesting their role in recovering endocytosed IRT1 (Ivanov et al., 2014). Intriguingly, the overexpression of MVB/LE-localized FREE1/FYVE1 (Gao et al., 2014) induces IRT1 accumulation at the PM (Barberon et al., 2014), implying that FREE1/FYVE1 may cooperate with SNX1 to facilitate IRT1 recycling to the PM. Unravelling how CLASP-microtubules and FREE1/FYVE1 regulate SNX1 and resolving the specific functions of Retromer and Retriever complexes will be important milestones to mechanistically understand how recycling to the PM is achieved in plants (Fig. 2).

5. Recycling of exocytic and vacuolar SNAREs

SNARE proteins are crucial regulators of specificity of vesicle-organelle and organelle-organelle membrane fusion. Preceding the actual fusion event, three Q-type SNAREs on the target membrane (t-SNAREs) form a *trans*-SNARE complex with the incoming R-type v-SNARE on the opposing membrane. After inducing membrane fusion, the *trans*-SNARE complex becomes a *cis*-SNARE complex that is disassembled by the NSF-ATP and SNAP to liberate individual SNAREs for use in the next round of vesicle transport. Reassembly of the SNARE complex is among other mechanisms prevented by recycling of v-SNAREs to their original membrane localization. Retrieval pathways for exocytic v-SNAREs are the most studied among the SNARE recycling processes in eukaryotes (Fig. 3). In contrast to PM receptors and transporters, internalized exocytic v-SNAREs are not recycled back to the cell surface but rather to the TGN to participate in novel rounds of exocytic trafficking. The family of ANTH domain-containing proteins functions as clathrin-associated sorting proteins (Zouhar and Sauer, 2014). Their role in v-SNARE recycling was described for all eukaryotic kingdoms. In yeast, ANTH protein family consists of three members. While all three yeast ANTH proteins participate in endocytic internalization of ubiquitinated cargo, only Yap1801p and Yap1802p (for yeast AP180 homologue) function in retrieval of Snc1p exocytic v-SNARE. In yeast

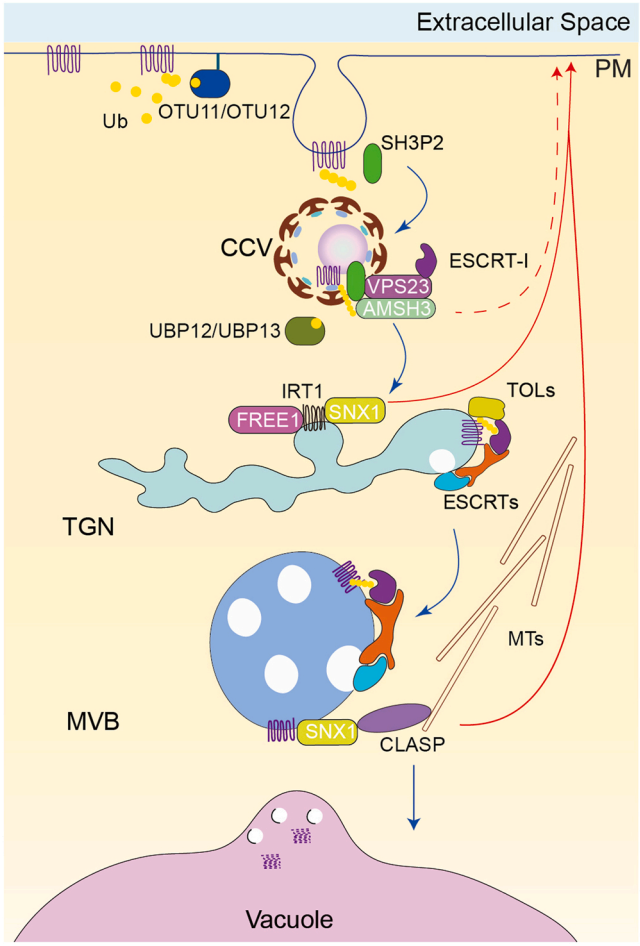

Fig. 2. Model of endocytic degradation and recycling pathways for PM proteins in plants. The covalent attachment of ubiquitin (Ub) to PM-localized cargo proteins initiates their internalization via the endocytic pathway (blue lines). SH3P2 binds the Ub chain of PM proteins and promotes their clathrin-mediated endocytosis. In CCVs, SH3P2 interacts with VPS23 to recruit ESCRT-I, which can then transfer the ubiquitinated cargo to downstream ESCRT machinery for their internalization in ILVs and their degradation in the vacuole. In this way, the ubiquitinated cargo proteins move through the TGN/EE, the MVB/PVC/LE, and are eventually delivered to the vacuole lumen for turnover. Deubiquitinating enzymes (DUBs) localized in different compartments can prevent protein degradation. In the PM, the DUBs OTU11 and OTU12 can deubiquitinate proteins and avoid their endocytosis. PM proteins can also be deubiquitinated after endocytosis to be retrieved back to the PM via recycling pathways (red lines). The DUB AMSH3 interacts with both SH3P2 and VPS23 and is recruited to the CCVs, where it can deubiquitinate cargo for their retrieval to the PM. In the TGN/EE, SNX1 coordinates with FREE1 to promote IRT1 recycling. In the MVB/PVC/LE, the microtubule (MT)-associated protein CLASP recruits SNX1 to retrieve endocytosed receptors to the PM.

yap1801/2 double mutant, vesicle-associated membrane protein (VAMP)/synaptobrevin homologue Snc1p accumulates at the plasma membrane, indicating that Snc1p recycling from the PM to the EEs is dependent on CCVs (Burston et al., 2009). In mammalian cells, two ANTH clathrin adaptors were identified as a functional homologues of yeast Yap1801/2 proteins in v-SNARE retrieval from the PM. CALM and AP180 adaptors mediate recycling of short-type VAMPs (brevins), including VAMP2, VAMP3 and VAMP8 (Koo et al., 2011; Miller et al., 2011). The long-type VAMPs (longins), like VAMP7, are retrieved from the PM via the interaction with HIV Rev-binding protein (Hrb), a clathrin adaptor and ArfGAP (Pryor et al., 2008). The Arabidopsis genome does not encode any Hrb homologues and the retrieval of longin-type v-SNAREs must be mediated by other mechanisms. In contrast to a

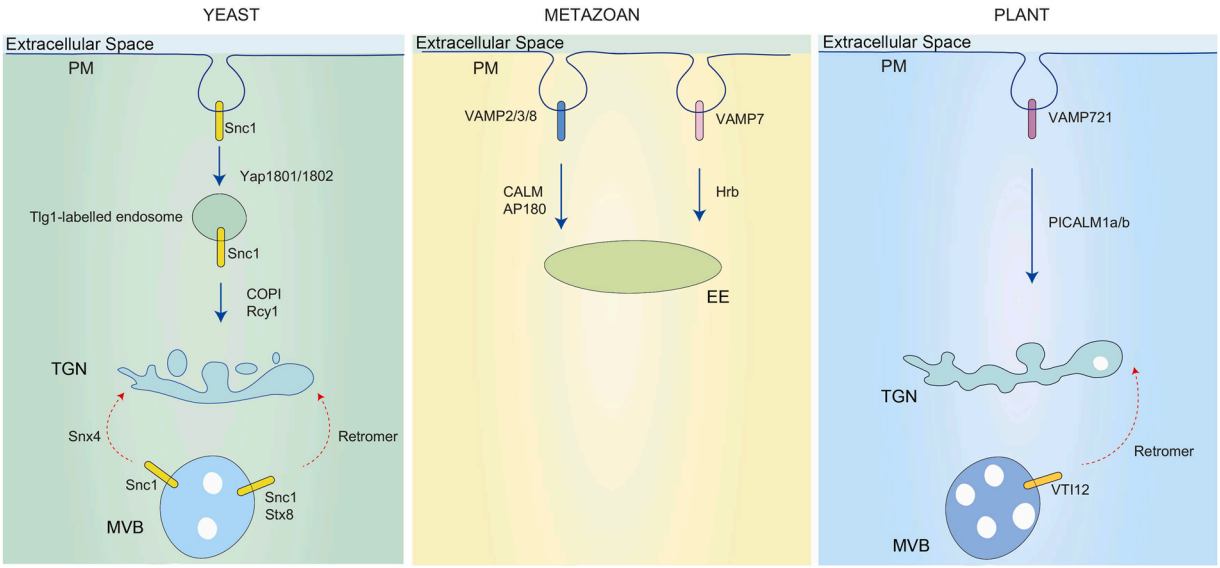

Fig. 3. Schematic of known SNARE recycling routes in yeast, metazoans and plants. In *Saccharomyces cerevisiae*, clathrin adaptors Yap1801p and Yap1802p mediate endocytosis of R-SNARE Snc1p to a Tlg1p-positive endosome, from where they are either selectively recycled by COPI/Rcy1 pathway back to the TGN or remain in the same membrane as it matures into MVB. From the late endosomal membrane, Snc1p can be retrieved to the TGN by either Retromer or Snx4p-dependent mechanisms. In *Schizosaccharomyces pombe*, Qc-SNARE Stx8p also utilizes Retromer for retrieval from the MVB to the TGN. In *Homo sapiens*, R-SNAREs from the brevin family (e.g., VAMP2/3/8) are recycled from the plasma membrane to the early endosome using CALM or AP180 clathrin adaptor proteins, while longins (e.g., VAMP7) use Hrb protein, a clathrin adaptor and ArfGAP. In *Arabidopsis thaliana*, R-SNARE VAMP721 utilizes ANTH proteins PICALM1a and PICALM1b for the TGN retrieval. Based on genetic and biochemical evidence, Qb-SNARE VTI12 recycling from the MVB depends on the Retromer complex.

small number of *ANTH* genes found in yeast and metazoan genomes, the plant ANTH proteins form a large and diverse family of 18 members, indicating a complexity in exocytic and endocytic pathways in plants (Zouhar and Sauer, 2014). Recently, the longin VAMP72 group was found to interact with PICALM1a/ECA1 and PICALM1b, two ANTH-domain proteins (Fujimoto et al., 2020). In the *picalm1a/b* double mutant, VAMP721 was accumulated at the plasma membrane and the amount of seed coat mucilage was substantially reduced (Fujimoto et al., 2020). Therefore, it was proposed that VAMP721 is retrieved to the TGN in PICALM1a/b-dependent manner, and that recycling of VAMP721 is essential for correct secretion of certain cargo, including the seed coat mucilage.

The endocytic vesicles carrying the retrieved v-SNAREs fuse with EEs. In this process, internalized exocytic v-SNAREs, such as VAMP8, function as R-SNAREs and bind their cognate Q-SNAREs at the early endosomal membrane (Antonin et al., 2000). From EEs, these internalized v-SNAREs may need to be transferred to the TGN to participate in new rounds of exocytic vesicle transport. The corresponding trafficking pathways were thoroughly investigated using yeast Snc1p as a marker for exocytic v-SNARE recycling. Up to date, three independent pathways have been proposed to mediate delivery of endocytosed Snc1p from endosomes to the Sec7p-labelled TGN membrane (Best et al., 2020). One pathway involves COPI vesicles, the phosphatidylserine flippase Drs2p and its activator F-box containing protein Rcy1p. Rcy1/Drs2/COPI mutants accumulate Snc1p in Tlg1p-labelled membranes of EEs (Hanamatsu et al., 2014; Xu et al., 2017; Best et al., 2020), suggesting that they are required for recycling internalized Snc1p from the EE to the TGN. In contrast, in yeast cells lacking endosome-localized sorting nexin Snx4p, Snc1p was detected in the limiting vacuolar membrane or inside the vacuole (Best et al., 2020), indicating that the Snx4p-mediated pathway recycles Snc1p from the MVB/LE to the Sec7p-labelled TGN, diverting it from degradation in the vacuole. Strikingly, in the double deletion mutant *rcy1Δ snx4Δ*, where retrieval pathways from early and late endosomes are both blocked, a small fraction of Snc1 was still competent for recycling. A complete block in Snc1p recycling, observed in triple mutant *snx4Δ rcy1Δ vps35Δ*, indicated that approximately 10% of the internal pool of Snc1p was recycled by the Retromer complex (Best et al., 2020). The role of Retromer in retrograde transport of SNAREs was also investigated for Stx8p, a Qc-SNARE and a homologue of mammalian syntaxin 8, from *Schizosaccharomyces pombe*. The Stx8p retrieval from LEs is mediated by Retromer and sorting nexin Snx3p, and requires a specific sorting motif (Yanguas and Valdivieso, 2021). This sorting determinant was conserved in fungal species close to *S. pombe*, but missing from syntaxin 8 homologues from metazoans and Arabidopsis (Yanguas and Valdivieso, 2021). In plants, the EE corresponds to the TGN so the internalized exocytic v-SNAREs can be directly reloaded in exocytic vesicles. Moreover, there are no reports on retrieval of exocytic v-SNAREs from MVBs in plants, but there is evidence that the Retromer and Retriever complexes recycle VTI-family SNAREs, which function in the vacuolar sorting pathway (Sanmartín et al., 2007). Arabidopsis plants carrying a point mutation in the *VTI11* SNARE gene display defects in amyloplast sedimentation in shoot endodermis, resulting in an agravitropic phenotype (Kato et al., 2002). Mutants in the Retromer subunits VPS26A, VPS29 and VPS35A suppress the gravitropism and morphological abnormalities of a *vti11* mutant (Hashiguchi et al., 2010). This suppression is most likely a result of the increased interaction between VTI12 and SYP2-family SNAREs observed in the Retromer mutants (Hashiguchi et al., 2010). In wild-type plants, the MVB-localized SYP2-family SNAREs interact preferentially with VTI11, whereas VTI12 interacts preferentially with TGN-localized SNAREs (Bassham et al., 2000; Sanderfoot et al., 2001). In the Retromer mutants, an impediment in retrieval of VTI12 would raise its levels at the MVB, which would allow for increased interaction with SYP2-family SNAREs, compensating for the absence of VTI11. Remarkably, mutants in *VPS26B* or *VPS35B/C* did not show suppression of *vti11* phenotypes (Hashiguchi et al., 2010). The specific role of the VPS26A-VPS29-VPS35A complex in the VTI12 retrograde transport may indicate a specialization in cargo retrieval for distinct combinations of Retromer subunits. Moreover, the Retriever complex may also be involved in recycling of VTI-family SNAREs. A mutation in the *VPS26C* gene suppresses the phenotypes of *vti13* (Jha et al., 2018), which include absence of a xyloglucan epitope in the cell wall and reduced root hair growth (Larson et al., 2014). This suggests that in the absence of a functional Retriever complex, certain components of vesicular trafficking machinery, possibly VTI11 or

VTI12, are not correctly recycled and their abnormal localization can compensate the *vti13* mutation. Further research on VTI-SNAREs as potential cargo of Retromer and Retriever may reveal how these complexes select their specific cargo and what precise transport pathways they are involved in

6. Recycling from the vacuole/lysosome

The vacuole/lysosome was considered until recently a final destination in the endomembrane system. In plants, fusion of the vacuole with the PM had been observed, but associated with programmed cell death (Hatsugai et al., 2009), which precludes any further possible recycling from the PM. However, recent results in yeast and metazoans suggest there is retrograde trafficking from the vacuole/lysosome. In yeast, Snx4p was shown to assemble at the vacuolar membrane and mediate Atg27p vacuole-to-endosome retrograde transport (Suzuki and Emr, 2018). In mammals, the AP-5 adaptor complex, which localizes in LEs and lysosomes, is involved in retrograde trafficking of CI-MPR from endolysosomes back to the TGN (Hirst et al., 2018; Seaman, 2019). In plants such pathways of retrieval from the vacuole have yet to be described. Homologues of three of the four subunits of the AP-5 complex have been identified in Arabidopsis thaliana (Law et al., 2022), but their subcellular localization and function remains uncharacterized, so it is an obvious gap to fill in future studies. A possible function of the plant AP-5 adaptor complex in retrieval of proteins from the tonoplast is an attractive hypothesis to explore.

CRediT authorship contribution statement

Jan Zouhar: Writing – review & editing. **Wenhan Cao**: Writing-review & editing. **Jinbo Shen**: Writing-review & editing. **Enrique Rojo:** Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

No data was used for the research described in the article.

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