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Receptor-mediated transport of vacuolar proteins: a critical analysis and a new model

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Abstract In this article we challenge the widely accepted view that receptors for soluble vacuolar proteins (VSRs) bind to their ligands at the *trans*-Golgi network (TGN) and transport this cargo via clathrin-coated vesicles (CCV) to a multivesicular prevacuolar compartment. This notion, which we term the "classical model" for vacuolar protein sorting, further assumes that low pH in the prevacuolar compartment causes VSR–ligand dissociation, resulting in a retromer-mediated retrieval of the VSRs to the TGN. We have carefully evaluated the literature with respect to morphology and function of the compartments involved, localization of key components of the sorting machinery, and conclude that there is little direct evidence in its favour. Firstly, unlike mammalian cells where the sorting receptor for lysosomal hydrolases recognizes its ligand in the TGN, the available data suggests that in plants VSRs interact with vacuolar cargo ligands already in the endoplasmic reticulum. Secondly, the evidence supporting the packaging of VSR–ligand complexes into CCV at the TGN is not conclusive. Thirdly, the prevacuolar compartment appears to have a pH unsuitable for VSR–ligand dissociation and lacks the retromer core and the sorting nexins needed for VSR recycling. We present an alternative model for protein sorting in the TGN that draws attention to the much overlooked role of Ca^{2+} in VSR–ligand interactions and which may possibly also be a factor in the sequestration of secretory proteins.

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Abbreviations

BFA	Brefeldin A
CCV	Clathrin-coated vesicle
ER	Endoplasmic reticulum
ESCRT	Endosomal sorting complex required for transport
LBD	Lumenal binding domain
MPR	Mannose 6-phosphate receptor
MVB	Multivesicular body
TGN	*trans*-Golgi network
VSR	Vacuolar sorting receptor

Introduction

With the recent publication by Kang et al. (2012) and the editorial comments given on it (Mach 2012), the impression gained by the reader is that anterograde transport of vacuolar sorting receptors (VSRs) together with their cargo ligands from the *trans*-Golgi network (TGN) to the prevacuolar compartment and the retromer-dependent recycling of ligand-free VSRs back to the TGN is an unchallenged fact. In the following, we would like to point out that far from being a foregone conclusion, VSR trafficking and the role of retromer in this process is by no means a clear-cut issue. Indeed, it remains most controversial with a number of problems needing to be addressed.

Selective transport of soluble proteins between compartments in the endomembrane system of eukaryotes requires membrane-spanning sorting receptors. These receptors fulfill dual functions: (1) they confer specificity for ligand recognition in the lumen of the donor compartment, and (2) they

physically link the ligands to cargo-selective coat proteins at the cytosolic surface. Coat proteins provide the mechanical force that deforms the membrane and simultaneously drive the formation of carriers and cargo loading (Faini et al. 2013). The release of the ligand from the sorting receptor in the acceptor compartment defines the end of the receptor-mediated transport route for the ligand with the receptor being then recycled back to the donor compartment for another round of transport. Donor- and acceptor compartments must provide differential conditions that trigger either formation or dissociation of receptor–ligand complexes: this is generally considered to be a question of pH and/or modulating divalent cations (Olson et al. 2008). Therefore, mechanisms must exist for the specific transport to and retention of H^{+}/Ca^{2+} transport complexes in the acceptor compartments.

In the simplest form, donor and acceptor compartments are persistent entities that communicate bidirectionally through vesicles, but even so it requires that ligands be delivered to the donor compartment, and at the same time being removed from the acceptor compartment. But what happens when the donor compartment is itself being continually formed, as is the case with the TGN, and the acceptor compartment continually being consumed as is the case with the prevacuolar compartment. How is receptor-mediated transport of ligands and receptor recycling achieved under such dynamic conditions?

VSRs and the "classical model" for receptor-mediated protein transport to the vacuole

The isolation of the transmembrane protein BP80 from a clathrin-coated vesicle (CCV) fraction from *Pisum sativum* that interacted in vitro with a peptide that carried the sequence-specific vacuolar sorting signal (asparagine–proline–isoleucine–arginine, NPIR) from proaleurain (Holwerda et al. 1992) was the first evidence that the sorting of soluble vacuolar proteins in plants is a receptor-mediated process (Kirsch et al. 1994). The genome of *Arabidopsis thaliana* encodes seven VSRs (AtVSR1, At3g52850; AtVSR2, At2g30290; AtVSR3, At2g14740; AtVSR4, At2g14720; AtVSR5 At2g34940; AtVSR6, At1g30900; and AtVSR7, At4g20110) (Shimada et al. 2003). With the exception of AtVSR2, which is expressed only in flowers, the other members of this protein family are expressed in all organs (Shimada et al. 2003) and it has been suggested that some of the VSRs are functionally redundant (Lee et al. 2013).

The pH dependency of interaction of BP80 with putative ligands in vitro (see below), together with its in situ localization to the *trans*-Golgi/ TGN stacks and prevacuolar compartments, resulted in the postulation of a model for receptor-mediated vacuolar transport and receptor recycling (Li et al. 2002; Paris et al. 1997). According to this model, which we will refer to hereafter as the "classical model", VSRs bind soluble vacuolar proteins in the TGN. This is then followed by the packaging of receptor–ligand complexes into CCVs, which are then transported to a prevacuolar compartment. There, the receptor–ligand complex dissociates, the receptors are recycled back via retromer-coated carriers to the TGN and the ligands are finally delivered to the vacuole through fusion of the prevacuolar compartment with the vacuole.

At the time, this model was in agreement with the widely accepted concept for mannose 6-phosphate receptor (MPR)-mediated transport of lysosomal proteins in mammals and with Vps10p-mediated vacuolar transport in yeast. The way stations en route to the lytic compartments seemed to be functionally homologous across the kingdoms. Advances have been made since then with respect to the functional and morphological characterization of compartments in the endomembrane system of plants. Firstly, the TGN itself is now recognized to act as an early endosome (Contento and Bassham 2012; Dettmer et al. 2006; Kang et al. 2011; Lam et al. 2009). Hence, it also receives internalized plasma membrane proteins via endocytic CCVs (Dhonukshe et al. 2007). The TGN/early endosome is therefore nowadays considered as the point of intersection between the biosynthetic vacuolar and the endocytic transport routes (Dettmer et al. 2006; Viotti et al. 2010). Secondly, the spherical, multivesicular body (MVB) has been identified as being the prevacuolar compartment lying downstream of the TGN and therefore constituting a late endosomal compartment (Tse et al. 2004). Thirdly, MVBs have been convincingly shown to fuse with the lytic vacuole, identifying them as transport carriers, rather than being persistent compartments on the vacuolar/endocytic pathways to the vacuole (Scheuring et al. 2011). VSR-mediated vacuolar protein transport must therefore be reexamined in the light of these recent discoveries.

The location(s) of VSRs

Although it has become a central dogma, there is actually no direct evidence to support the claim that ligand-free VSRs are transported in a retrograde manner from the prevacuolar compartment to the TGN. This interpretation finds its origin in the observation, especially in tobacco cells, that VSRs, but also non-functional chimeras with green fluorescent protein (GFP) substituting for the luminal ligand binding domain, often accumulate in the prevacuolar compartment (Tse et al. 2004; daSilva et al. 2005; Kim et al. 2010). As an explanation for the predominant location of VSRs to the prevacuolar compartment, Li et al. (2002) hypothesized that "VSR proteins only recycle back to the Golgi briefly for selection of transit cargo molecules and then return to the prevacuolar compartment for cargo delivery". Not only is this highly speculative, but with the ERD2 receptor, we have just the opposite situation. This receptor which recognizes K(H)DEL-proteins in the *cis*-Golgi

and returns them to the endoplasmic reticulum (ER) is, under steady-state conditions, mainly present in the *cis*-Golgi, where it binds its ligands. The receptor is detectable in the ER, where the ligands are released, and from which compartment it recycles, but at much lower levels (Griffiths et al. 1994). This distribution pattern is conserved in mammals (Griffiths et al. 1994) and plants (Brandizzi et al. 2002), and reflects the mechanistic principle of this essential sorting step. This receptor filters ER resident proteins out of the default secretory stream by providing a barrier that awaits the arrival of ligands. However, in yeast, the steady-state distribution of ERD2 can be shifted from the binding- to the recycling compartment by the overproduction of ligands (Lewis and Pelham 1992). Together, this suggests that the steady-state distribution of a sorting receptor is not a reliable indicator for defining the location of receptor recycling.

The claim that the distribution of VSRs in plants is similar to that of MPRs in mammalian cells (Li et al. 2002) was based on a report by Griffiths et al. (1988). However, a more detailed immunogold study has revealed similar levels of both types of MPR at the TGN and early endosome (Klumperman et al. 1993). More recently, it was shown that the bulk of transiently expressed GFP cation-independent MPR locates to the TGN (Waguri et al. 2003). More significantly, immunogold electron microscopy of endogenous VSRs in plants shows that they are just as detectable at the TGN as at the prevacuolar compartment (Sanderfoot et al. 1998; Hinz et al. 2007; Niemes et al. 2010b; Viotti et al. 2010; Richter et al. 2007). Such a bimodal distribution is of course much more in keeping with a situation where receptors cycle between two compartments. However, it also raises the question whether the VSRs that accumulate in the prevacuolar compartment, a situation that is seen especially in tobacco, are physiologically functional, i.e. will recycle, or whether they reflect a population of non-functional receptors which are destined for degradation in the vacuole.

The observation that the steady-state distribution of transiently expressed VSRs in tobacco protoplasts could be shifted upstream from the prevacuolar compartment to the TGN/EE supports the latter possibility. This happens without blocking the transport of soluble cargo ligands to the vacuole when mutants of the sorting nexins were expressed over the short term (Niemes et al. 2010b). Vacuolar delivery of soluble cargo would not be possible under these conditions if a receptor-mediated sorting step between the TGN/early endosome and the prevacuolar compartment was mandatory. This is, however, in direct contradiction with results that latrunculin B treatment also caused VSRs to relocate to the TGN/early endosome (Kim et al. 2005). This drug is often used to depolymerize actin microfilaments (Morton et al. 2000), and under these conditions, anterograde transport of soluble vacuolar cargo was blocked at the TGN/early endosome. Curiously, the latrunculin B treatment did not affect the transport of soluble (invertase:GFP) and membrane (H^+-ATPase:GFP) proteins to the plasma membrane (Kim et al. 2005). This is surprising considering the numerous reports which show that secretory vesicle transport from the Golgi to the plasma membrane is dependent on actin microfilaments in plants (Vidali and Hepler 2001; Robinson 1977).

VSRs have long been thought to be restricted to the compartments of the endomembrane system of plant cells, but there are several reports where endogenous VSRs have been immunologically detected at the plasma membrane as well (Laval et al. 1999; Wang et al. 2011). These data have been significantly substantiated by Saint-Jean et al. (2010) using *Arabidopsis* lines stably expressing a fluorescent functional VSR construct (citrine-AtVSR4). The plasma membrane was clearly labelled in “upper root cells” in these lines, and upon application of brefeldin A (BFA), the fluorescent AtVSR4 label entered “BFA bodies”. This drug is well known to target some Golgi/TGN-localized ADP-ribosylation factor–guanine nucleotide exchange factors causing the TGNs to separate from the Golgi stacks and to form aggregates, termed BFA bodies (Langhans et al. 2011). Wash out of BFA in the presence of the protein synthesis inhibitor cycloheximide led to recovery of the AtVSR4 signals at the plasma membrane. These data strongly suggest that AtVSR4, like the PIN family of auxin transporters (Grunewald and Friml 2010), is constitutively internalized and recycled back to the plasma membrane. Since the BFA body is mainly composed of TGN elements, a scenario was proposed whereby missorted VSR–ligands might be retrieved from the cell surface and were then transported via CCVs to the TGN/early endosome where they would be released (Saint-Jean et al. 2010). This, however, constitutes an indirect challenge to the classical model for secretory vacuolar protein transport, since recycling of VSRs from the TGN/early endosome back to the plasma membrane for further uptake of ligands requires the release of ligands in the TGN/early endosome. For this to work, one would need different sets of VSRs, each having opposing requirements (pH or otherwise) for their ligand interactions. For the first set, the conditions in the TGN/early endosome would have to cause the dissociation of the ligand, while the same conditions would have to promote high-affinity binding of ligands for the second set of VSRs. Significantly, this contrasts with the situation in mammalian cells where both types of MPRs (one for biosynthetic transport at the TGN, the other for internalization at the plasma membrane) transport ligands to the same location, the early endosome, which obviously provides a suitable environment for ligands to dissociate from both types of MPRs. Nevertheless, an important consequence of the findings from Saint-Jean et al. (2010) is that the VSRs in the isolated CCV fraction originally used for the identification of BP80 (Kirsch et al. 1994) may have been of plasma membrane (i.e. endocytic) rather than TGN origin, thereby questioning their primary role in biosynthetic vacuolar protein transport.

Clathrin and biosynthetic vacuolar protein transport

CCVs are easily recognized by their polyhedral coat, which consists of cargo-specific adaptor complexes, accessory proteins and clathrin (Brodsky 2012). In this process, cargo (a term often used for receptor–ligand complexes) is recognized and concentrated by adaptor complexes, which in turn recruit clathrin triskelia (homotrimers of the clathrin heavy chain, which can also associate with clathrin light chains). To date, five adaptor complexes, two clathrin heavy and two clathrin light chains have been described in mammals, allowing for specific sorting and transport events by CCVs at multiple locations (Brodsky 2012). Most genes encoding the clathrin machinery are conserved in *Arabidopsis* (Chen et al. 2011), and despite the compartmental differences between mammals and plants, CCVs are also formed at the plasma membrane and the TGN/early endosome of higher plants. While it is now established that CCVs at the plasma membrane are carriers for receptor-mediated endocytosis (Dhonukshe et al. 2007), the function of TGN/early endosome-derived CCVs is less clear. Are they involved in anterograde traffic of VSR–ligand complexes from TGN/early endosome to the prevacuolar compartment as presumed according to the classical model? Do they instead specifically recycle receptors from the plasma membrane, or do they even perform both functions, implying that there must be two distinct populations of CCV at the TGN/early endosome.

One approach to test for CCV-mediated transport is through manipulation of clathrin cage formation. A commonly used tool to achieve this is the expression of a dominant-negative clathrin heavy chain mutant: the clathrin hub fragment (Liu et al. 1998). The hub fragments titrate out the clathrin light chains leading to reduced levels of assembly-competent hexameric triskelions, thereby inhibiting clathrin light chain-dependent CCV formation. Surprisingly, the hub fragment can be constitutively expressed in plant cell cultures, without lethal effects (Adam et al. 2012; Tahara et al. 2007). Nevertheless, and even though transient expression of the hub fragment has been successfully used to block clathrin-mediated endocytosis in *Arabidopsis* protoplasts (Dhonukshe et al. 2007; Scheuring et al. 2011; Chen et al. 2011; Kitakura et al. 2011), this mutant did not prevent the soluble vacuolar cargo reporter RFP-AFVY from reaching the vacuole (Scheuring et al. 2011). This can only be explained by assuming that a CCV-mediated transport step is not mandatory for cargo to reach the vacuole.

There is, however, a possible caveat with hub expression experiments. Mammalian cells possess two genes encoding clathrin heavy chains, which exhibit 85 % identity in their protein sequences, but only one of the two is associated with the clathrin light chains (Brodsky 2012). Hub expression would therefore not be effective in inhibiting a CCV-mediated transport process, which uses trimeric rather than hexameric CHC triskelions. The *Arabidopsis* genome also encodes for two clathrin heavy chains, but since they are 98 % identical, it appears to be very likely that they both bind clathrin light chains and that this association is required for the formation of all CCVs, even if they might mediate transport from different locations. In this regard, it was recently shown that *Arabidopsis* clathrin heavy chains are functionally redundant, although clathrin heavy chain 1 knockout lines were more defective in endocytic uptake of the styryl dye FM4-64 than the clathrin heavy chain 2 knockout lines (Kitakura et al. 2011).

Another way to influence the levels of functional hexameric triskelions is by generating clathrin light chain mutants. There are three clathrin light chains in *Arabidopsis* (Scheele and Holstein 2002), whereby the *clc1* knockout is lethal (Wang et al. 2013a). In contrast, *clc2/clc3* double knockout mutants are viable but have only a slightly reduced vacuolar delivery of PIN2-GFP (Wang et al. 2013a). However, since PIN2-GFP is endocytosed at the plasma membrane prior to vacuolar delivery, it is not possible to ascertain whether the inhibited CCV-dependent transport step lies at the plasma membrane or at the TGN/early endosome.

An alternative but more indirect approach to study the contribution of CCVs to vacuolar sorting is to analyse the transport of mutated VSRs, which lack positive sorting information for the interaction with adaptor complexes and thus cannot to be incorporated into CCVs. These mutants usually have amino acid substitutions within the tyrosine-based sorting motif (YXXΦ, with Φ representing a bulky hydrophobic amino acid) in the cytosolic tail of the VSR (Ohno et al. 1998). This motif is also thought to be recognized by the μ subunits of adaptor complexes in plants (Happel et al. 2004) linking them to clathrin triskelions, which triggers CCV formation. Interestingly, a mutated VSR that carried a Y/A exchange was found to localize mainly to the TGN/early endosome and to the plasma membrane rather than to the prevacuolar compartment in tobacco epidermal cells (daSilva et al. 2006). This mis-localization was explained by the lack of an efficient CCV-mediated TGN to prevacuolar compartment transport step.

The cytosolic tail of VSRs contains further motifs, potentially allowing for multiple targeting and transport steps. For example, in stably transformed *Arabidopsis* plants citrine-AtVSR4 localized to the plasma membrane and was present as punctate structures, possibly the TGN, due to endocytic recycling (Saint-Jean et al. 2010). The endocytic transport of this receptor from the plasma membrane depends on the amino acid sequence isoleucine–methionine (IM) positioned upstream of the tyrosine-based sorting signal and is conserved in all VSRs. This motif (ExxxIM) was suggested to act as an endocytosis signal similar to the dileucine signal ([D/E]xxxL[L/I/M]) in mammals (Saint-Jean et al. 2010). Therefore, one could say that endocytic CCVs, which carry VSRs, can also contribute to vacuolar transport.

Finally, it has been shown that the AtVSR1 undergoes a homotypic interaction via its C-terminus and that this interaction is required for its localization to the prevacuolar compartment (Kim et al. 2010). By the use of alanine substitution mutagenesis, a stretch of nine amino acids upstream of the tyrosine motif (with the ninth amino acid being the tyrosine of the tyrosine-based motif), was found to be required for this homotypic interaction. The resulting mutant (C2A) also failed to reach the prevacuolar compartment and instead localized to the Golgi, as demonstrated by immunofluorescence co-localization with the Golgi stack markers mannosidase 1-GFP and Lewis epitope-containing glycans (Fitchette et al. 1999). Significantly, when the C2A mutant was transiently expressed in *Arabidopsis* leaf protoplasts the vacuolar cargo reporter aleurain-GFP was secreted. Co-immunoprecipitation of the ligand aleurain-GFP with either AtVSR1-HA or AtVSR1-C2A-HA revealed a higher intracellular accumulation of ligands with the C2A receptor mutant line as compared to the wild type (Kim et al. 2010). However, immunoprecipitation of the wild-type (AtVSR1:HA) and mutant (AtVSR1-C2A:HA) receptors from CCV fractions isolated from the wild-type and mutant lines, respectively, revealed considerably lower amounts of the C2A mutant receptor in the isolated CCV fractions (Kim et al. 2010). While these results do indeed suggest that the abrogation of vacuolar protein transport in the C2A mutant is due to an inability to incorporate VSR–ligand complexes into CCVs, they also point to the fact that the analysis of receptor localization and cargo transport can be severely perturbed due to heterotypic interactions, occurring between wild-type VSRs and mutagenized VSRs, carrying single amino acid substitutions in their cytosolic tail.

Very recently, four papers were published, which address the role of CCV in post-Golgi trafficking in plants. They all deal with the effects of mutant adaptors. In one of the papers, a mutant of a monomeric EPSIN-like adaptor protein called MTV1 was shown to have minor effects on the transport of vacuolar proteins (Sauer et al. 2013). The authors therefore interpreted their results as supporting the notion that CCV are required for vacuolar protein transport. A more severe effect on vacuolar protein transport was observed when a mutant of the μ-adaptin (AP1M2) belonging to the tetrameric AP-1 adaptor complex was expressed (Park et al. 2013). However, in addition a serious affect on the secretion of a non-vacuolar reporter was recorded. Moreover, in *ap1m2* mutant plants, trafficking of the syntaxin KNOLLE to the cell plate was impaired. The latter effect was confirmed in a third paper (Teh et al. 2013). Most significantly, the same mutant was also defective in PIN2 recycling to the plasma membrane (Wang et al. 2013b). Thus, we have the situation that inhibition of AP-1 adaptor function results in negative effects on multiple post-Golgi transport routes. As such, and since adaptors are supposed to be specific for only one transport route, the data presented in these papers cannot be taken as unequivocal evidence for the participation of CCVs in the export of vacuolar proteins out of the TGN. A more detailed discussion of these papers is to be found elsewhere (Robinson and Pimpl 2013).

Where do VSRs first meet their cargo ligands?

According to the classical model for the transport of newly synthesized vacuolar proteins, the binding of VSRs to soluble cargo ligands occurs in the TGN as in mammalian cells. However, one must be aware that in mammals the receptor-recognition motifs for soluble lysosomal cargo molecules are added to lysosomal acid hydrolases in the *cis*-Golgi but they remain cryptic until becoming demasked in the TGN immediately prior to MPR binding (see Robinson et al. (2012) for a more complete description). As a result of this intricate mechanism, lysosomal acid hydrolases do not represent true MPR–ligands until their sorting signals become exposed. This principle has two major consequences: firstly, the transport of soluble proteins to the lysosome cannot bypass the TGN and, secondly, MPR-mediated sorting cannot occur in a compartment earlier than the TGN.

In sharp contrast, vacuolar sorting signals of soluble proteins in plants are strictly encoded in their amino acid sequence. Most of these signals consist of only short amino acid sequence motifs, e.g. NPIR, which are often present at their N- or C-termini. No post-translational modification has yet been identified that either confers vacuolar targeting of a secretory protein or is required for the vacuolar delivery of VSS-containing proteins (Neuhaus and Rogers 1998; Robinson et al. 2005). Therefore, soluble vacuolar cargo molecules are already ligands for VSRs immediately after their co-translational insertion into the lumen of the ER, which of course is where VSRs themselves are synthesized. Consequently, if VSR-mediated sorting of vacuolar proteins in plants occurs according to the classical model and thus requires receptor–ligand interaction in the TGN/EE, one must ask about the mechanism(s) which prevent receptor–ligand interactions from taking place upstream of this compartment. This problem has hitherto not been adequately addressed. On the contrary, there is mounting evidence pointing to the fact that VSR–ligand interactions occur very early in the secretory pathway.

PV72 is a VSR in the membrane of so-called precursor accumulating vesicles of developing pumpkin (*Cucurbita pepo*) cotyledons (Shimada et al. 1997). These vesicles bud off the ER and carry storage proteins (mainly 2S albumin and 11S globulin) directly to the protein storage vacuoles, i.e. circumventing the Golgi apparatus (Hara-Hishimura et al. 1993). PV72 binds to both the NPIR sorting signal of aleurain as well as to pro2S albumin via a C-terminus located vacuolar sorting signal (Shimada et al. 2002). A soluble, ER-targeted

derivative of PV72 was generated that consisted of the luminal binding domain (LBD) of this receptor fused to the ER-retrieval motif HDEL (PV72-HDEL (Watanabe et al. 2004)). In a transgenic *Arabidopsis* line stably expressing this construct, the PV72-HDEL was shown to reside in the ER and to specifically co-accumulate the 41-kDa precursor of aleurain. This demonstrates that the ER-targeted soluble receptor prevents vacuolar arrival and thus processing of proaleurain into the 28-kDa mature form. A similar ER-targeted VSR derivative was generated by daSilva et al. (2005) which consisted of the luminal binding domain of *Arabidopsis* VSR4 fused to an HDEL sequence. The transport of the vacuolar NPIR-containing reporter amylase-sporamin was then investigated by transient co-expression in tobacco mesophyll protoplasts. Under these conditions, the reporter was seen to accumulate in a microsomal (i.e. ER-containing) fraction rather than being found in the vacuole. However, even though both receptor–HDEL constructs influenced vacuolar transport by binding ligands in the early secretory pathway, it is difficult to define precisely the location of their interaction since it is unclear whether the HDEL–receptor-mediated retrieval is restricted to the *cis*-Golgi cisternae or might also occur from cisternae more distal in the stack. Nevertheless, these data also show that whereas the ligand binding domain of the VSR is sufficient for the initial ligand binding, the transmembrane domain and the cytosolic tail seem to be required for subsequent targeting (Brandizzi et al. 2002; Tse et al. 2004; Saint-Jean et al. 2010).

Direct evidence for ligand binding by VSRs in the lumen of the ER has been provided using two different strategies (Niemes et al. 2010a). In the first case, the LBD of AtVSR4 was fused to the transmembrane domain and the cytosolic tail of the ER-resident chaperone calnexin, which effectively anchored the LBD in the lumen of the ER. In the second instance, a fluorescent protein was fused to the relatively short cytosolic tail of the wild-type VSR, which caused steric hindrance of ER export. In both cases, co-expression of these chimeras in tobacco leaf protoplasts with the soluble fluorescent vacuolar reporters aleurain-GFP or GFP-sporamin resulted in their co-accumulation in the ER (Niemes et al. 2010a). More recently *vsr1/vsr3* and *vsr1/vsr4* double knockout mutants were generated to analyse transport to the lytic vacuole in *Arabidopsis* protoplasts (Lee et al. 2013). The transiently expressed fluorescent vacuolar reporters *Arabidopsis* aleurain-like protein-GFP or phaseolin-GFP were missorted and localized to the ER, with some amounts of the cargos being secreted. If VSRs were to interact with their ligands only in the TGN/early endosome, as assumed in the classical model, the transport of the vacuolar cargo towards the TGN/early endosome would occur via non-selective bulk-flow together with secretory cargo and would thus be independent of VSRs. Consequently, the lack of functional VSRs in the mutants should not perturb arrival of both cargos in the TGN. However, the vacuolar reporters were not detected in either the Golgi or the TGN, but instead accumulated in the ER. This clearly indicates that VSRs are required for efficient cargo export out of the ER.

The notion that VSR-mediated sorting starts in the ER is further supported by an important feature of PV72. This receptor has a consensus sequence for Ca^{2+}-binding in one of the epidermal growth factor repeats (cbEGF) located within the C-terminus of the luminal domain. Ca^{2+}-binding to PV72 leads to a conformational change in the luminal domain of the receptor, which forms a pocket that can bind ligands (Watanabe et al. 2002). This resembles the situation with the well-characterized LDL receptor at the surface of hepatocytes whose extracellular domain also possesses a cbEGF Ca^{2+}-binding domain that is required for stabilizing the receptor–ligand complex (Fass et al. 1997). The EC_{50} for the Ca^{2+}-dependent interaction with PV72 is 40 μM (Watanabe et al. 2002). This concentration is well exceeded by measured values for Ca^{2+} in the ER lumen of mammalian cells (Montero et al. 1997). The ER of plant cells is also assumed to have a high Ca^{2+} content as a result of the presence of Ca^{2+}-ATPases (Bonza and De Michelis 2011) and due to the requirements of Ca^{2+}-binding chaperones (Christensen et al. 2010).

Location and recruitment of retromer and the sorting nexins

Receptor-mediated sorting of soluble vacuolar proteins necessitates efficient recycling of the VSRs. In the case of the vacuolar/lysosomal sorting receptors in yeasts and mammals, this occurs via a protein complex called "retromer" (Seaman 2005; McGough and Cullen 2011). Retromer has two main subunits: the heterotrimeric retromer core, also termed cargo recognition complex, which consists of VPS35, VPS29 and VPS26, and the sorting nexin proteins. The sorting nexin proteins are Vps5p and Vps17p (Horazdovsky et al. 1997) and SNX1/2 and/or SNX5/6, in yeast and mammalian cells, respectively (Cullen and Korswagen 2012). In the process of retromer-mediated recycling, the retromer core interacts with the cytoplasmic domain of the sorting receptors via the VPS35 subunit and the sorting nexins bind to the retromer core and to phospholipids and thus drive membrane tubularization. In this way, cargo selection is linked to the formation of tubular transport carriers (Hierro et al. 2007). Proteins homologous to the retromer core forming proteins have been described in plants (Oliviusson et al. 2006) but it seems that the *Arabidopsis* genome lacks a VPS17 homologue and instead encodes for three sorting nexins (SNX1, SNX2a and SNX 2b, (Jaillais et al. 2006)).

The concept of retromer-based recycling of VSRs was incorporated into the concept of VSR-mediated sorting on

the basis of the demonstration that antibodies against VPS35 can co-immunoprecipitate VSRs (Oliviusson et al. 2006). Accordingly, the correct location of retromer is crucial to understanding the mechanism(s) of VSR recycling. Unfortunately, the original allocation of the three retromer core subunits to the prevacuolar compartment (Oliviusson et al. 2006) was subsequently shown to be incorrect having been a consequence of insufficient blocking in double immunofluorescent labelling experiments (Niemes et al. 2010b). In contrast, in single immunofluorescent labelling studies on transgenic tobacco BY-2 cells stably expressing fluorescent organelle markers, VPS29 clearly locates to the TGN/early endosome and not the prevacuolar compartment (Niemes et al. 2010b). Immunogold electron microscopy of *Arabidopsis* root cells has confirmed this location for endogenous VPS29 and also showed that VPS29 was present in the core of brefeldin A bodies which represent aggregates of the TGN (Niemes et al. 2010b; Langhans et al. 2011).

Controversy has also surrounded the subcellular location of the plant sorting nexins (see also Robinson et al. (2012) and Stierhof et al. (2013) for a detailed discussion), but the situation seems to have been resolved with convincing immunogold labelling of the TGN/early endosome, but not the MVB/prevacuolar compartment for both SNX1 and SNX2a (Stierhof et al. 2013). Together, the ultrastructural localization of both the retromer core and the sorting nexin proteins at the TGN/early endosome strongly argues for the TGN as the starting point for retromer-mediated recycling for VSRs. It also implies that VSR-mediated transport of ligands begins at a location upstream of the TGN. Consequently, post-TGN transport of soluble vacuolar cargo must occur independently of VSRs, which recycle from the TGN back to an upstream location for another round of ligand binding.

In agreement with this suggestion is the observation that long-term suppression of sorting nexin function, either through sorting nexin mutant expression or RNAi knockdown, impairs VSR-mediated transport to the lytic vacuole at an upstream location of the TGN, rather than at a downstream location. In these cases, VSR–ligands but also newly synthesized VSRs finally failed to be exported from the ER and accumulated at this upstream location (Niemes et al. 2010a), a phenotype which has also been observed for soluble vacuolar cargo under VSR knockout conditions (Lee et al. 2013). This ER-export failure is specific for vacuolar cargo but does not apply to secretory cargo (Niemes et al. 2010a) and indicates that retromer-mediated recycling exhibits a feed-back control on ER export for vacuolar cargo. Therefore, it is plausible to assume that retromer-mediated recycling and ER-export of VSRs and ligands represent two linked transport routes. This is similar to the situation observed for the COP-I and COP-II pathways in ER–Golgi vesicle trafficking. Here, the specific inhibition of the COP-I-mediated retrograde recycling route results in the collapse of the COP-II-mediated anterograde ER-export route (Stefano et al. 2006).

Recent advances have also been made with respect to deciphering the mechanism of retromer assembly. As previously shown for mammalian cells (Rojas et al. 2007), there is now evidence that the plant retromer complex does not exist as a fully assembled pentameric complex in the cytosol which is then recruited en bloc to the membrane (Pourcher et al. 2010). It seems that the recruitment of the sorting nexins and the retromer core complex occurs independently of each other, with recruitment of the sorting nexins not being a prerequisite for the attachment of the retromer core to membranes (Pourcher et al. 2010). Recruitment of the retromer core occurs first through attachment of VPS35 (Zelazny et al. 2013).

VPS35 recruitment is mediated by a Rab GTPase: RABG3f, which belongs to the Rab7 family of GTPases. In mammalian cells, the retromer core recruitment is regulated by a switch from Rab5 to Rab7 GTPases on maturing endosomal membranes (Seaman et al. 2009). Plants have eight Rab7 family members (Nielsen et al. 2008), seven of which locate to the tonoplast, and this is also the case for RABG3f, which is commonly used as one of the “Wave” marker lines (Geldner et al. 2009). In contrast to both of these reports where the fluorescent signal for (X)FP-tagged RABG3f was evenly distributed throughout the tonoplast, the co-localizing signals for GFP-RABG3f and mCherry-VPS35a in the report from Zelazny et al. (2013) were distinctly punctuate. Therefore, exactly which compartment is being labeled is unclear. However, if RABG3f does bind to the tonoplast this would also place the retromer core at this location and, as a consequence, would mean that the sorting nexins and the retromer core lie at different ends of the vacuolar trafficking pathway: one at the TGN the other at the tonoplast. Not only is this in contrast to the situation in both yeast and in mammalian cells, where there is no evidence for retromer-mediated receptor recycling from the vacuole or lysosome, but it is also difficult to understand how the attachment of only the retromer core can drive tubularization of the spherical, turgescent vacuolar membrane without the involvement of the sorting nexins, in particular since the retromer core—at least in yeasts—has an alpha-solenoid structure that is recruited to already tubularized membranes (Hierro et al. 2007). Alone, the retromer core cannot cause tubularization. Clearly, more work is required to solve this paradoxical situation.

Is retromer function required for the transport of all vacuolar proteins?

Despite differential modes of retromer assembly in yeasts, mammals and plants, it also appears as if there are differential sorting nexin requirements for retromer core-mediated receptor recycling in plants, dependent on the type of cargo and the

developmental situation of the cells (Zelazny et al. 2013). While it is assumed that sorting nexin function is required for efficient VSR-mediated transport of soluble proteins to the lytic vacuole (Niemes et al. 2010a; Kang et al. 2012), it has been questioned whether sorting nexin function is mandatory for the efficient transport and delivery of storage proteins to the protein storage vacuole (Zelazny et al. 2013), even though this transport might be mediated by the same VSRs (Lee et al. 2013; Zouhar et al. 2010; Shimada et al. 2006). The effects on the transport of the storage proteins 12S globulin and 2S albumin was investigated in maturing embryos of *Arabidopsis* sorting nexin loss-of-function mutants (Zelazny et al. 2013). Interestingly, there was a differential effect with the transport of 12S globulins requiring sorting nexin function but not in the transport of the 2S albumin. This contrasts with the observation that loss-of-function of VPS29 or VPS35 blocked transport of both proteins to the protein storage vacuole and resulted in their secretion (Yamazaki et al. 2008).

Based on the assumption that retromer-mediated recycling is indirectly required for correctly functioning anterograde transport, it was postulated that “sorting nexins work with the core retromer for 12S globulin trafficking but are dispensable for 2S albumin maturation, with the latter relying only on a functional core retromer” (Pourcher et al. 2010). However, it has to be considered that the two storage proteins might traffic differently: whereas the globulins are transported through the Golgi apparatus and MVB/prevacuolar compartment (Robinson and Hinz 1999) and may indeed need retromer to recycle their VSRs, 2S albumin—as discussed above—is sorted via the VSR PV72, which directs the 2S albumin into an ER-derived precursor-accumulating vesicle that bypasses the Golgi on its way to the protein storage vacuole (Hara-Nishimura et al. 1998, 2004). There is to date no evidence for receptor recycling in this trafficking route, at least not in pumpkin cotyledons, as the PV72-2S albumin complex probably does not dissociate in the vacuole due to high Ca^{2+} concentrations in their lumen (Hara-Nishimura and Shimada 2006). Therefore, in respect to the function of PV72 in the transport of 2S albumin transport, retromer-mediated recycling of PV72 seems unlikely to occur.

Retromer and VSR recycling from the prevacuolar compartment?

An L(Φ)/A exchange in the tyrosine motif of a VSR reporter not only resulted in its enhanced arrival at the tonoplast but also caused partial co-localization with a Venus-Rha1-positive compartment, which was subsequently termed late prevacuolar compartment (Foresti et al. 2010). This mis-localization was explained on the basis that the mutation of the tyrosine-based sorting signal did not prevent CCV-mediated anterograde transport to the prevacuolar compartment but interfered instead with retromer-mediated recycling from the prevacuolar compartment (daSilva et al. 2006). However, since a VSR reporter lacking the entire cytosolic tail (and therefore unable to interact with clathrin heavy chains or for that matter retromer) also trafficked to the vacuole (daSilva et al. 2006). At best, all that these results tell us is that a tyrosine-based motif in the cytosolic tail of a VSR is not a strict requirement for reaching the vacuolar route, but that the cytosolic tail is required for VSR recycling. They constitute neither direct proof for VSR–clathrin interactions at the TGN/early endosome nor for retromer-mediated VSR recycling from the prevacuolar compartment.

The role of retromer in vacuolar protein transport in *maigo1-1* plants has been recently investigated (Kang et al. 2012). These mutants carry a T-DNA insertion of 57 bases downstream of the stop codon of the *VSP29* coding sequence in the 3′-untranslated region. This does not eliminate VPS29 entirely but results in an 8.4-fold reduction of the transcript level of VPS29 compared to wild-type plants (Shimada et al. 2006). In contrast to wild-type plants (see also Kim et al. 2005), the relative proportion of VSRs in the prevacuolar compartment in *mag1-1* plants remained unaffected by latrunculin B treatment. This observation led to a contention that “VSR1:HA does not efficiently travel from the prevacuolar compartment to the TGN in *mag1-1* plants” (Kang et al. 2012) implying that impaired retromer function was responsible for this. Curiously, this defect seems to be without repercussion on VSR distribution since it is identical in both wild-type and *mag1-1* plants, i.e. with over 80 % in the prevacuolar compartment in each case (Kang et al. 2012). The authors also performed transient expression with the soluble vacuolar reporters aleurain-GFP and sporamin-GFP in leaf protoplasts isolated from the *mag1-1* plants and claimed that the trafficking of soluble proteins to the vacuole was inhibited in *mag1-1* mutant plants, with the vacuolar reporters being trapped in the TGN. A closer look at the data provided, however, reveals that the fluorescent signals in the vacuoles and the detection of the vacuolar degradation product of the GFP-based cargos are almost indistinguishable between *mag1-1* and wild-type protoplasts. The postulated “significant” differences might have resulted from the unusual classification/definition of vacuolar and non-vacuolar cargo distribution, since “protoplasts showing a vacuolar staining pattern with three or less than three punctate stains per protoplast were considered to have a vacuolar pattern, whereas protoplasts with more than four punctate stains per protoplast were considered to have a non-vacuolar pattern, even if they showed a weak vacuolar staining pattern”. Since the location of the retromer core was not actually demonstrated in this paper (Kang et al. 2012), its possible function in recycling VSRs from the MVB/prevacuolar compartment remains at best speculative.

The prevacuolar compartment: is it a recycling compartment at all?

The lack of evidence in favour of the MVB/prevacuolar compartment being a compartment where VSR–ligand dissociation occurs begs the question as to whether the prevacuolar compartment is at all a compartment from which recycling takes place? Studies on the recycling of the PIN family of auxin transporters suggested that PIN1 could be retrieved from the prevacuolar compartment in order to enter the constitutive recycling pathway which starts from early and recycling endosomes (Grunewald and Friml 2010). This interpretation was based on the premise that SNX1 and the retromer subunit VPS29 localized to the MVB/prevacuolar compartment. However, as discussed above, these proteins locate instead to the TGN. More recently, a direct trafficking route from the MVB/prevacuolar compartment to the plasma membrane has been proposed which utilizes the Rab GTPase ARA6 (known to locate to the MVB; Spitzer et al. 2009) and the Q-SNARE VAMP727 which is present at the plasma membrane (Ebine et al. 2012). However, an interaction between ARA6 and VAMP727 may in fact reflect the direct fusion of MVBs with the plasma membrane rather than implying a vesicular transport process.

The problem with this suggestion, as with all claims for recycling or even transport out of persistent MVBs/prevacuolar compartments in general, is that this is not supported by the ultrastructural properties of these organelles. Even in rapid frozen specimens, MVBs are more or less spherical entities and lack vesiculation or tubularized profiles, which would count as evidence for the occurrence of an export out of this compartment through a membranous carrier (Robinson et al. 2012; Stierhof et al. 2013). Therefore, the observed morphology of the MVBs/prevacuolar compartments is not as what one would expect if bidirectional vesicle transport were in operation between the TGN and the MVB/prevacuolar compartment especially since vesiculation at the TGN is readily observable in electron micrographs.

Is the prevacuolar compartment a compartment where VSRs and ligands can dissociate?

A prerequisite for the supposed recycling of VSRs from the prevacuolar compartment is that this is indeed the compartment en route to the vacuole where vacuolar cargo ligands dissociate from the VSRs. pH is a crucial factor for the coordinated transport of MPRs and cell surface receptors in mammalian cells (Maxfield and McGraw 2004). Failure to release ligands after endocytic uptake inhibits the recycling of the receptor and results in their lysosomal degradation (Davis et al. 1987). Most receptors release ligands at a lower pH than they bind, but some receptors require a lower pH than others, and this determines the endosomal compartment in which they separate (Maeda et al. 2002). An exception to this rule however seems to be the KDEL receptor ERD2, which binds ligands at a pH that is lower than the pH in the compartment for the release (Wilson et al. 1993). This variation might be an adaptation of the sorting mechanism according to the pH of the compartments at which the sorting occurs, since the *cis*-Golgi-localized KDEL receptor recycles escaped soluble ER-resident proteins against an acidifying pH gradient back to the ER (Wilson et al. 1993). In contrast to ERD2, MPRs sort ligands along an acidifying pH gradient.

The binding of peptide ligands by BP80 from pea also appears to be pH-dependent. One in vitro binding study has shown that there is a broad binding optimum at pH 6 that falls off to 50 % of the maximum at pH 7.5 and at pH 5.0 (Kirsch et al. 1994). In another study, PV72 was seen to bind the NPIR-containing ligand at pH 7 (Watanabe et al. 2002). According to the "classical" model for secretory vacuolar protein transport, the pH at the plant TGN, which represents the binding compartment, should be higher than that of the prevacuolar compartment, the dissociation compartment. Do the available data fit this scenario?

Attempts to measure the pH in the TGN and MVB/prevacuolar compartment of plant cells using organelle markers fused to pH-sensitive pHluorins have recently been performed. The expression of such pH sensors in *Arabidopsis* protoplasts has resulted in pH values of 6.5±0.2 for the TGN/early endosome and 6.2±0.4 for the MVB/prevacuolar compartment (Shen et al. 2013). When transiently expressed in leaf epidermal cells, these reporters have given pH values of 6.1 for the TGN and 6.6 for the MVB/prevacuolar compartment (N. Paris, personal communication). From these data, it is clear that the pH in the MVB/prevacuolar compartment is not significantly more acidic than in the TGN; it may even be more alkaline. This finding is not surprising considering the lack of detectability of proton pumping complexes like VHA-a1 ATPase and PPase in the MVB/prevacuolar compartment (Dettmer et al. 2006; Robinson et al. 2012; K. Schumacher, personal communication). However, we would have predicted a significantly lower pH for the TGN than was measured. As was previously pointed out (Robinson et al. 2012), the pH in the TGN/EE should be more acidic than at the cell surface in order for internalized receptor–ligand complexes from the plasma membrane to dissociate. The pH at the plasma membrane–cell wall interface (i.e. the apoplast) is between 5.5 and 6 (Gao et al. 2004; Monshausen et al. 2009, 2011) and this corresponds to the pH binding optimum for receptor–ligand complexes, e.g. FLS2 and flg22 at the plasma membrane or the apoplast, respectively (Robatzek and Wirthmueller 2013), so that a pH in the TGN lower than this might have been expected. Having said that, it must be also be pointed out that, despite numerous examples for receptor-mediated uptake of extracellular ligands, e.g. BRI1 (the brassinosteroid receptor)

and BR (brassinosteroid hormone), there is currently no evidence for the dissociation of the ligand from the receptor in the plant TGN/early endosome. Indeed, the maintenance of the receptor–ligand interaction in the early endosome is the basis for continued signal transduction from this compartment (Geldner and Robatzek 2008; Irani et al. 2012).

The TGN and the MVB/prevacuolar compartment: stable, long-living organelles or transitory, maturing compartments in a membrane continuum from the Golgi to the vacuole?

It is implicit in the classicalmodel for VSR trafficking that the TGN and the prevacuolar compartment are distinct organelles which constantly communicate through bidirectional vesicle transport. In support of this model is the observation that when *trans*-Golgi (ST-XFP) and TGN (XFP-SYP61) markers are co-expressed they consistently label two distinct and separate organelle populations with no apparent overlap. This phenotype also exists for TGN markers and "prevacuolar compartment-resident VSRs" (De Marcos Lousa et al. 2012; Foresti and Denecke 2008). Due to this apparent lack of intermediate stages between the Golgi stack and the TGN and between the TGN and the prevacuolar compartment, it was concluded that these organelles are separate entities and that transport between them can only occur through vesicles (De Marcos Lousa et al. 2012; Foresti and Denecke 2008). Despite the fact that the size of transport vesicles (40–80 nm diameter) lies below the resolution of light microscopy, it must be taken into account that these data reflect a situation after the marker molecules have reached their steady state distribution in long-term expression experiments. If the distribution is analysed at earlier stages, it becomes clear that fluorescent VSR signals (GFP:BP-80) initially co-localize with TGN marker signals (SYP61-RFP), but then gradually separate as the length of the expression period increases (see Supplemental Fig. 4 in Scheuring et al. 2011). This suggests that newly synthesized VSRs move through the TGN on their way to the prevacuolar compartment, but are not accompanied by SYP61, indicating a degree of selectivity for this transport step, with some molecules being retained in the TGN while others are transported further in an anterograde direction.

Selectivity in regard to the transport of membrane proteins can of course be achieved by transport vesicles due to a specific interaction with coat proteins during vesicle formation (Balch et al. 1994). The observation that a single point mutation (Y612A) in the cytoplasmic tail of BP-80 causes a shift in the steady state distribution of the VSR to the TGN (daSilva et al. 2006) has therefore been used as an example in support of the selectivity in transport that can be imparted by vesicle formation (De Marcos Lousa et al. 2012). It has furthermore been claimed (De Marcos Lousa et al. 2012) that such a selective transport event is incompatible with the notion that the TGN matures into the MVB/prevacuolar compartment (Scheuring et al. 2011). However, it is difficult to understand why the requirement for specificity and the process of maturation should be mutually exclusive, since the specific transport to the lysosome via early to late endosomes in mammalian cells is generally considered to occur via a highly dynamic maturation process, which does not involve transport vesicles (Rink et al. 2005; van Weering et al. 2010). In our opinion, specificity alone cannot be the key determinant for a particular transport mode, but rather the dynamics, function and morphology of the compartments must also be taken into consideration. It should also be pointed out that the endocytic route to the lysosome via early and late endosomes does not merge with the secretory route from the TGN to the plasma membrane in mammalian cells. Therefore, in mammals, TGN-derived transport vesicles (CCV) are required to connect these otherwise independent routes. In plants, these routes merge at the TGN. Therefore, it is questionable to consider transport vesicles as a strict requirement for TGN/early endosome to MVB/prevacuolar compartment transport in plants.

Despite this controversy, several important questions need to be addressed: (1) How do the TGNs and the prevacuolar compartments arise? (2) Are they subjected to any kind of turnover? (3) Are they consumed in the process of sorting and transport? These are questions which have not yet been addressed in the context of the classical model for VSR trafficking. The available data indicate that the TGN is derived from the Golgi stack and its existence is a consequence of cisternal maturation in a *cis-trans* direction. This first became apparent in early electron micrographs of the Golgi apparatus in maize root cap cells, where cisternae were seen leaving the Golgi stack—a process then termed "sloughing" (Mollenhauer and Morre 1991; Kang et al. 2011; Toyooka et al. 2009).

The dynamics of cisternal release from the Golgi stack has recently been visualized by live cell imaging in hypocotyl cells from an *Arabidopsis* line expressing fluorescent markers for the stack (ST-GFP) and for the TGN (VHAa1- RFP) (Viotti et al. 2010). Surprisingly, not only did the TGN behave as an organelle independent to the Golgi stack but occasionally reassociated with it. It can be inferred that the TGN is a product of the Golgi stack. This is based on the observation that concanamycin A, a specific inhibitor of V-type ATPases (Dettmer et al. 2006), prevents the release of the TGN and lead to an increase in the number of cisternae per stack, with TGN markers then being found in the *trans*-most cisternae of the stack (Viotti et al. 2010).

Despite its significance, an answer to the question about the lifespan of a TGN is still elusive. While the observed reassociation with Golgi stacks (Viotti et al. 2010) supports the idea of a long-living discrete compartment. An EM-tomographical analysis suggests that the TGN fragments into

secretory vesicles, CCVs and tubular remnants shortly after dissociation from the Golgi stack (Kang et al. 2011). Provided that the TGN is formed and disintegrated continually, a fragmentation-based sorting mechanism would obviate the need for ongoing CCV-mediated anterograde transport to the MVB as proposed in the classicalmodel. Moreover, fragmentation-based sorting at the TGN would also have severe consequences for the recycling of the VSRs: A TGN that dissociates from the stack and disintegrates represents a transport carrier that contains only a limited amount of cargo for secretion and vacuolar transport and obviously would be without further cargo supply from the Golgi after its release from the stack. Therefore, it seems questionable whether this same TGN can represent the target compartment for recycling VSRs as is predicted in the classicalmodel. However, if the TGNs were indeed the target compartment for VSR recycling under these conditions, additional mechanisms must exist that enable MVB-derived recycling carriers to sense cargo levels in the lumen of the TGN, which prevent recycling of VSRs to a TGN that is short on ligands. On the contrary, an unlimited supply of ligands for recycled VSRs and therefore the ultimate target of the VSR recycling route should be the location where ligand binding occurs, and this target seems to lie in the early secretory pathway (daSilva et al. 2006; Niemes et al. 2010a; Watanabe et al. 2004). Therefore, it appears that fragmentation-based sorting of the TGN (Kang et al. 2011) would also be in best agreement with retromer-mediated VSR recycling from rather than to the TGN.

The puzzling question however is as to whether anterograde transport of vacuolar cargo from the TGN strictly requires a vesicle shuttle? It has been reported that the prevacuolar compartments develop out of the TGN by a budding process (Scheuring et al. 2011). Although intermediate stages of this process are rarely seen, more frequent examples have been recorded in cells recovering from concanamycin A treatment (Scheuring et al. 2011). This suggests that TGN-MVB maturation is a rapid and continuous process. Concanamycin A treatment also leads to a rapid and drastic reduction in the number of MVBs/ prevacuolar compartments in the cell (Scheuring et al. 2011) since further generation of MVBs is prevented whereas fusion of MVBs/prevacuolar compartments appears to continue unperturbed. This assumption seems to be plausible, since this fusion event should be independent of TGN function and thus will still occur in the presence of the drug. The evidence for Golgi–TGN–prevacuolar compartment maturation presented in Scheuring et al. (2011) is also in agreement with the observed localization of the ESCRT complexes. ESCRT protein complexes sort ubiquitinated transmembrane proteins into the intraluminal vesicles of MVBs thus enabling them to be degraded when the MVB fuses with the lysosome/vacuole (Hurley 2008). Ubiquitination occurs at the plasma membrane and proteins tagged with this signal are internalized via CCV and travel down the endocytic pathway to the MVBs. Thus, ESCRT-mediated formation of intraluminal vesicles beginning at the TGN is fully in agreement with the concept of MVB budding. However, the function of the plant TGN in secretion, vacuolar protein transport and endocytosis makes it conceptually difficult to understand how sorting of the three different types of cargo is achieved in this organelle.

An alternative model for the separation of secretory and vacuolar cargo in the TGN

As we have described above, a cornerstone of the classicalmodel for the VSR-mediated sorting of soluble vacuolar cargo proteins is the pH dependency of the VSR–ligand interaction. This is however seriously challenged in the key paper of Watanabe et al. (2002) who stated that "The association and dissociation of PV72 with the ligand is modulated by the Ca^{2+} concentration rather than the environmental pH". Indeed, the authors showed that ligands remain attached to PV72 in the presence of 50 μM Ca^{2+}, even at pH 4 (Watanabe et al. 2002). Thus, it has to be considered that binding and release of ligands is mainly modulated by the availability of Ca^{2+}. In this regard, high concentrations of Ca^{2+} could promote binding in unfavourable pH conditions but also prevent premature release during a gradual pH decrease along a given transport route. Conversely, pH may well be important under low Ca^{2+} conditions. We have presented evidence and argued the case for VSRs interacting with their cargo ligands in the ER, where the Ca^{2+} concentration is higher than in the cytosol. The concentration of Ca^{2+} is even higher in the vacuole (Conn et al. 2011; Stael et al. 2012), while the pH gradually decreases from the ER via the Golgi towards the vacuole (Shen et al. 2013). If dissociation of the VSR–ligand complex requires low Ca^{2+}, as proposed by Watanabe et al. (2002), but also low pH, where in the endomembrane system could such conditions be encountered? Moreover, what consequences would this have for sorting mechanisms other than the VSR-mediated cargo?

It is implicit in the classical model for VSR-mediated vacuolar protein transport that default secretion to the plasma membrane occurs by via bulk flow. While this term was originally introduced to differentiate between selective (sorting signal requiring) and non-selective (without sorting signals) transport of proteins (Pfeffer and Rothman 1987), it was deduced from this term that secretory cargo molecules simply move into secretory vesicles by bulk flow. Recently, this simplistic view has now given way to a novel concept according to which secretory proteins are actively sorted into secretory vesicles at the TGN of mammalian cells through a process that is dependent on Ca^{2+} (von Blume et al. 2009). This mechanism involves the cytosolic actin-severing proteins ADF and cofilin on the cytoplasmic side of the TGN, the Ca^{2+}-ATPase SPCA1 (in yeast, Pmr1p) in the TGN membrane, and the Ca^{2+}-binding protein Cab45 in the lumen of the TGN (Curwin et al. 2012; von Blume et al. 2011; Scherer

et al. 1996). It was suggested that severing of actin filaments at the surface of the TGN through ADF/cofilin provides the physical requirements that allow for the formation of vesicular carriers but also activates the TGN-localized Ca^{2+} pump SPCA1 in this domain of the TGN (von Blume et al. 2009). Sequestration of secretory cargo in the emerging vesicles is supposed to occur via Cab45, which binds not only to SPCA1 but also to secretory proteins. This interaction is strongly dependent on the Ca^{2+}-binding sites within the six EF-hand motifs of Cab45, since a Cab45 mutant, which lacks Ca^{2+}-binding capability, can neither bind to SPCA1 nor to secretory proteins (von Blume et al. 2012). Perturbation of the Ca^{2+} concentration by SPCA1 knockdown significantly decreased the secretion of secretory proteins, indicating a positive requirement for Ca^{2+} in secretion. However, lysosomal proteins such as Cathepsin D are secreted under Ca^{2+} depletion conditions (von Blume et al. 2012). These data not only suggest that Cab45 might act as a mediator directing soluble proteins into TGN domains that are destined to form secretory vesicles, but it also shows that TGN-based lysosomal sorting via MPRs is influenced by Ca^{2+} levels.

Because this Ca^{2+}-based mechanism operates in both yeast and mammalian cells, it has been suggested that it is evolutionarily conserved, i.e. is likely to be present in plants as well (Curwin et al. 2012). However, the molecular functions of Ca^{2+} in protein sorting and transport processes within the plant secretory pathway are largely unknown. *Arabidopsis* contains four calcium ATPases of the P_{2A} class (endoplasmic reticulum-type calcium ATPase; ECA1-4) and ten calcium ATPases of the P_{2B} class (autoinhibited calcium ATPase; ACA) (Baxter et al. 2003). While AtECA1 and AtECA2 seem to localize to the ER (Hong et al. 1999), and AtACA4 localizes to the tonoplast (Geisler et al. 2000), it seems that AtECA3 localizes to the Golgi/TGN/endosomes (Mills et al. 2008; Li et al. 2008).

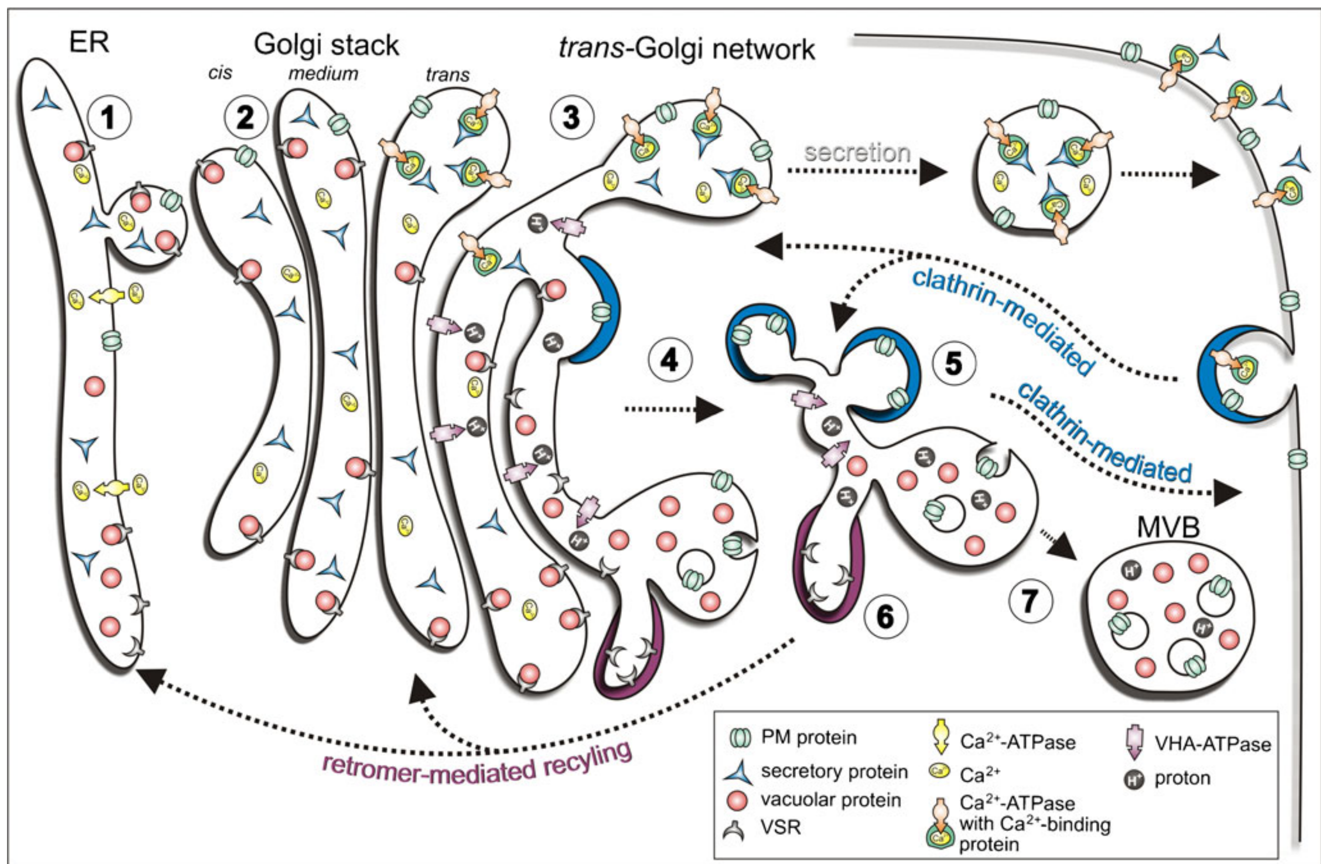

Fig. 1 A Ca^{2+}-based model for the sorting of vacuolar and secretory proteins in the TGN. *1*—High concentrations of Ca^{2+} in the ER lumen cause soluble vacuolar cargo ligands to bind to VSRs. These, together with secretory proteins, exit the ER in COP-II-coated vesicles which then fuse with the *cis*-cisternae of an adjacent Golgi stack. *2*—Secretory and vacuolar cargo, the latter still bound to their receptors move downstream through the Golgi stack as a consequence of cisternal maturation. *3*—In the early *trans*-Golgi network (TGN), the activity of a second Ca^{2+}-ATPase again leads to high Ca^{2+} concentrations. These conditions ensure that the VSR–vacuolar ligand interaction remains intact, but also cause soluble secretory proteins to bind to a Cab45-like Ca^{2+}-activated protein leading to their sequestration in secretory vesicles. *4*—In the late TGN, characterized by the H^+ pumping VHA-ATPase but lacking the Ca^{2+}-ATPase, an environment conducive to CSR–ligand dissociation is achieved. This late TGN compartment is then released from the Golgi stack. *5*—Clathrin-coated vesicles (CCV) are formed at one domain of this free TGN, and recycle internalized plasma membrane proteins. *6*—Retromer-coated carriers with ligand-free VSRs are formed at another domain. *7*—The residual domain of the TGN then transforms into a multivesicular body that contains soluble vacuolar cargo ligands

Intriguingly, *eca3* mutants seem to be impaired in the sorting of apoplastic peroxidases (Li et al. 2008).

If indeed a Ca^{2+} pump is required as a membrane anchor for the secretion of soluble proteins, must this pump be a Golgi/TGN localized protein? As we have discussed above for the various receptors, the steady-state distribution of a sorting receptor should not be used as the sole indicator for its function or its transport mode. The concept of Ca^{2+}-mediated cargo selection (von Blume et al. 2012) implies that the calcium pump is an integral part of the secretory vesicle after fission. This would suggest that this key player of the sorting machinery is also transported to the plasma membrane. It also means that the source of increasing Ca^{2+} concentration would be continually depleted during the sorting process. In turn, this would require a recycling step from the plasma membrane to the TGN in order to return the Ca^{2+} pump for further sorting. However, if indeed a high concentration of Ca^{2+} is required for secretion at the plant TGN, this would suggest that this process also entails a separation of these sorting processes. This is because, in that part of the TGN where secretory cargo exits, vacuolar cargo cannot do so because it will remain attached to the VSRs due to the high concentration of Ca^{2+}. This being so, dissociation of vacuolar cargo ligands must occur downstream of the exit of the secretory cargo. This is in full agreement with the postulated formation of secretory vesicles already seen at the *trans*-most cisternae of the Golgi, which mature into the TGN (Kang et al. 2011). The continuous early sorting of secretory cargo in the TGN and also removal of Ca^{2+} pumps would generate an environment that would constantly favour vacuolar sorting. In this respect, it might even be plausible to assume that this early sorting is the driving force for the maturation of the *trans*-most cisternae into a TGN and would represent the first alteration in membrane composition compared to the stack. Early removal of secretory cargo at the *trans*-most cisternae of the Golgi would imply that the corresponding recycling route from the plasma membrane would also lead to this location. This assumption is also be in agreement with the suggested function of this compartment, since it was shown that the *trans*-most cisternae already assumes the function of an early endosome (Kang et al. 2011). Indeed,

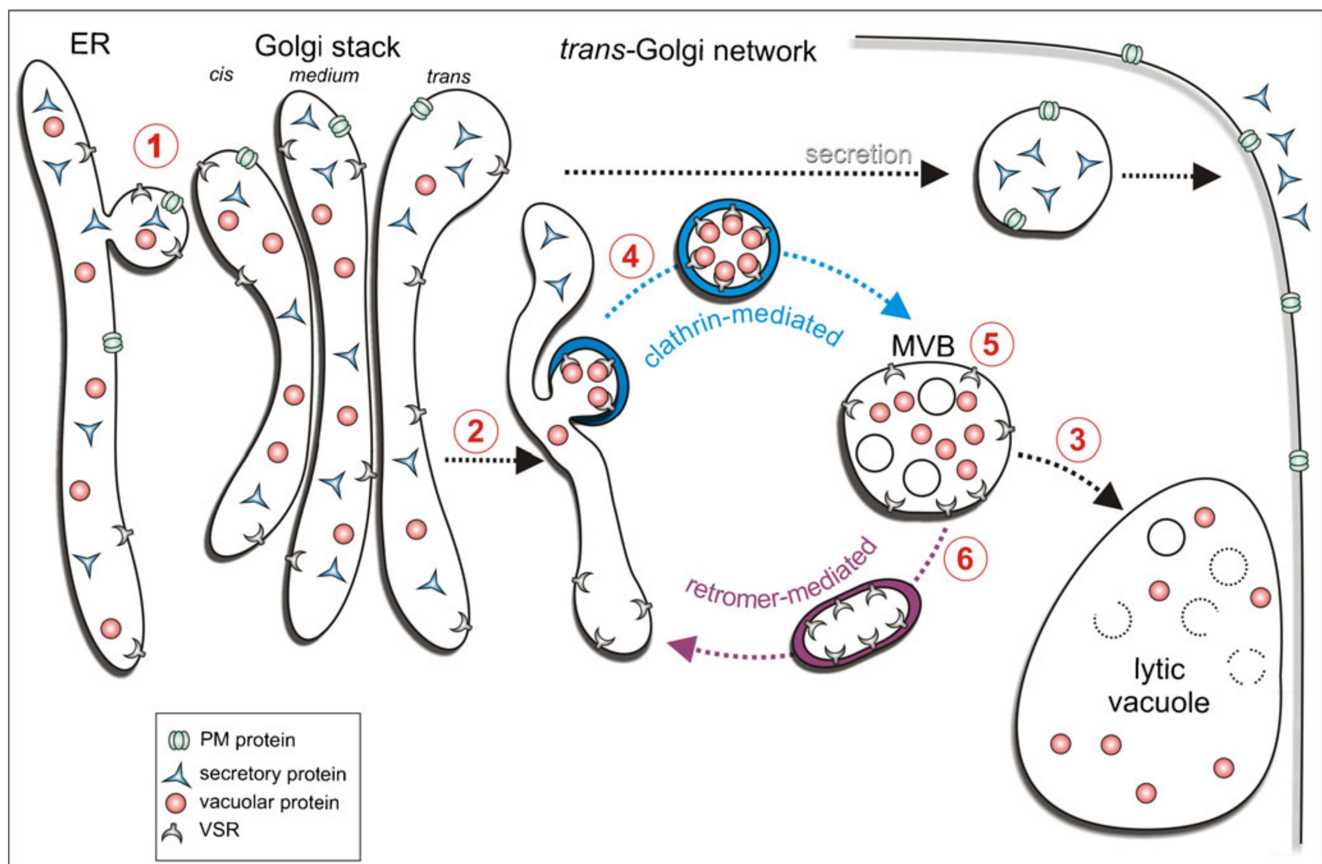

Fig. 2 The weak points in the classical model for VSR-mediated transport of soluble vacuolar cargo in the endomembrane system of plants. The weak points are indicated by *numbers circled in red* in the cartoon. *1*—What prevents VSR–ligand interactions occurring earlier than the TGN in the secretory pathway? *2*—The origin and maintenance of membrane equilibrium in the TGN is unclear. *3*—If the MVB fuses with the lytic vacuole, how is it replaced? *4*—Is there only one class of CCV formed at the TGN? What about CCV-mediated recycling to the plasma membrane? *5*—The pH in the MVB is not suitable for VSR–ligand disscociation. *6*—Neither the sorting nexins nor the retromercore locate to the MVB

endosomal transport of the dye FM4-64 to the *trans*-most cisternae has been suggested as a mechanism that explains the delivery of the endocytosed dye to the newly forming cell plate during cytokinesis (Kang et al. 2011).

An important aspect of the proposed Ca^{2+}-based sorting model is the notion that secretory proteins seem to be linked to a calcium pump by a soluble calcium-binding protein. This raises the question as to whether the pump does indeed increase the free Ca^{2+} concentration in the TGN. It is possible that the calcium-binding protein binds Ca^{2+} immediately after translocation into the lumen. This could mean that the TGN does not necessarily have to possess high levels of free Ca^{2+}. In this scenario, the VHA-H^+-ATPase would lower the pH of the TGN relative to the Golgi, which might then be sufficiently acidic to cause dissociation of the VSR ligand (see Fig. 1).

Conclusions

As we have seen, the generally accepted dogma that VSRs (a) pick up soluble cargo ligands at the TGN and (b) sequesters them into CCVs, and then (c) transports them to the prevacuolar compartment, from which compartment, (d) the ligand-free VSRs are recycled back to the TGN via retromer-coated carriers, does not withstand a critical analysis of the available data. In fact, there are so many contradictory observations which mean that it is difficult to uphold this classical model for (receptor-mediated vacuolar transport, see Fig. 2).

A simpler concept of TGN-based sorting of secretory and vacuolar proteins was put forward by us in a previous review (Robinson et al. 2012), but this was done without knowledge of measured pH values in the endomembrane system of plants and lacking proper appreciation of the role of Ca^{2+}. We have now elaborated on this concept by proposing that VSR–ligand dissociation occurs under low Ca^{2+}/low pH conditions in a “late” TGN compartment after secretory proteins have exited the *trans*-Golgi/TGN under conditions of high Ca^{2+}. Obviously, much work needs to done before this concept can be verified. Ca^{2+} concentrations in the subdomains of the TGN will need to be determined, Ca^{2+}-binding proteins in the TGN must be identified and localized, and of course the precise location and properties of the responsible Ca^{2+}-ATPases established. However, a good initial step forward would be the clear demonstration by high-resolution immunogold electron microscopy that, in contrast to soluble vacuolar cargo, secretory proteins are not detected in late TGN subdomains.

Interesting developments in the field of vacuolar protein sorting in plants will surely be forthcoming in the near future and we hope that this review will have acted as a stimulant in this regard.

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Conflict of interest The authors declare that they have no conflict of interest.

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