**The Aspergillus nidulans syntaxin PepA<sup>Pep12</sup> is regulated by two Sec1/Munc-18 proteins to mediate fusion events at early endosomes, late endosomes and vacuoles**

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Syntaxins are target-SNAREs that crucially contribute to determine membrane compartment identity. Three syntaxins, Tlg2p, Pep12p and Vam3p organize the yeast endovacuolar system. Remarkably filamentous fungi lack the equivalent of the yeast vacuolar syntaxin Vam3p, leaving open how these organisms regulate vacuole fusion. We show that the nearly essential *A. nidulans* syntaxin PepA_Pep12, present in all endocytic compartments between early endosomes and vacuoles, shares features of Vam3p and Pep12p, being capable of forming compositional equivalents of all known yeast endovacuolar SNARE bundles including that formed by yeast Vam3p for vacuolar fusion. Our data further indicate that regulation by two Sec1/Munc-18 proteins, Vps45 in early endosomes, and Vps33 in early and late endosomes/vacuoles contributes to the wide domain of PepA_Pep12 action. The syntaxin TlgB_Tlg2 localizing to the TGN appears to mediate retrograde traffic connecting post-Golgi (sorting) endosomes with the TGN. TlgB_Tlg2 is dispensable for growth but becomes essential if the early Golgi syntaxin SedV_Sed5 is compromised, showing that the Golgi can function with a single syntaxin, SedV_Sed5. Remarkably, its pattern of associations with endosomal SNAREs is consistent with SedV_Sed5 playing roles in retrograde pathway(s) connecting endocytic compartments downstream of the post-Golgi endosome with the Golgi, besides more conventional intra-Golgi roles.
Introduction

SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins are key players of membrane fusion. Anchored on opposing membranes, four SNARE domains (the characteristic ~70-residue consensus motif of these proteins) form a tetrameric complex bringing membranes in close proximity, thereby facilitating fusion (Jahn & Scheller, 2006). Structurally, this tetramer consists of highly stable bundle of four α-helices connecting donor and acceptor membranes as a 'trans-SNARE complex' or ‘SNAREpin’ (Sutton et al., 1998). The progressive zippering of the SNAREpin generates the force that catalyzes fusion (Sudhof & Rothman, 2009).

The four α-helices of the SNAREpin are highly intertwined, forming sixteen stacked layers of interacting side chains, all layers being essentially hydrophobic excepting the central ‘zero-layer’, which includes one arginine (R) and three glutamine (Q) chains (Fasshauer et al., 1998, Jahn & Scheller, 2006, Kloepper et al., 2007). Thus SNAREs are structurally classified as R- and Q-SNAREs according to the side chain that they contribute to the ‘zero layer’, with Q-SNAREs being further subdivided into Qa, Qb and Qc classes (Jahn & Scheller, 2006). Each SNARE complex is composed of Qa, Qb, Qc and R partners. The R-SNARE usually corresponds to the v-SNARE (‘incoming’ vesicle-SNARE), and the Q-SNAREs usually correspond to the t-SNAREs (target-SNAREs). SNARE complexes have organelle-specific composition and indeed some function in a unique step. However, others are more promiscuous, both in their localization and in their partners, implying that additional players must contribute to dictate the specificity of membrane fusion. For example, Sec1/Munc-18 (SM) proteins are compartment- and SNAREpin-specific ‘clasps’ that bind SNARE bundles to promote their fusogenic action (Sudhof & Rothman, 2009).

An additional layer of specificity is provided by RAB GTPases, which regulate SNAREs through their ‘effectors’. For example, in the case of the endocytic pathway, the CORVET (class C core vacuole/endosome tethering) and HOPS (homotypic fusion and vacuole protein sorting) multi-component vesicle tethering complexes (MTCs), which are recruited by RAB5 and RAB7 to early endosomes (EE) and late endosomes (LEs), respectively, coordinate membrane tethering with SNAREpin assembly and
fusion. This is in part achieved by integrating the SM protein Vps33 as a component of these complexes (Epp et al., 2011, Kummel & Ungermann, 2014).

Qa-SNAREs, also denoted syntaxins, are compartment-specific t-SNAREs. Besides the SNARE domain, syntaxins contain an intramolecular bundle of three helices denoted the Habc domain and an N-terminal ~20-residue peptide, specific for each syntaxin. The cognate SM protein that regulates the SNAREpin(s) often binds this syntaxin N-terminal peptide (Misura et al., 2000, Dulubova et al., 2002, Burkhardt et al., 2008, Yamaguchi et al., 2002).

The A. nidulans genome encodes 19 SNAREs (Gupta & Heath, 2002, Sánchez-Ferrero & Peñalva, 2006). Contrasting with the 24-SNARE repertoire of S. cerevisiae (Burri & Lithgow, 2004), the SNARE repertoire of A. nidulans includes single orthologues of the duplicated yeast sso1/sso2 and snc1/snc2 pairs, denoted ssoA and synA, respectively (Taheri-Talesh et al., 2008). However, the most remarkable difference between S. cerevisiae and A. nidulans, which is actually in common with other filamentous fungi, is the absence in the latter of an orthologue of the Vam3p syntaxin (Gupta & Heath, 2002, Sánchez-Ferrero & Peñalva, 2006). In S. cerevisiae Vam3p is the Qa-SNARE required for fusion between late endosomes and vacuoles, and between vacuoles themselves (Sato et al., 1998, Ungermann et al., 1998, Nichols et al., 1997, Pelham, 1999). Phylogenetic analysis indicates that Pep12p and Vam3p arose from duplication of an ancestral Qa SNARE that took place in the Saccharomycotina only, suggesting that the roles of Vam3p are played by a ‘broad range’ endosomal syntaxin in filamentous fungi of the Pezizomycotina lineage (including Aspergillus)(Kienle et al., 2009). Thus A. nidulans thrives with only five syntaxins. Of these the ER syntaxin Ufe1 has not been studied, SsoA sso1 acts in the plasma membrane (Taheri-Talesh et al., 2008), SedV Sed5 acts in the early Golgi (Pantazopoulou & Peñalva, 2011) and TlgB Tlg2, localizes to the late Golgi (TGN) (Pinar et al., 2013). Notably, the fifth, that we denoted PepA Pep12, is present in endosomes and vacuoles (Findon et al., 2010, Abenza et al., 2012), consistent with our proposal that PepA Pep12 assumes roles of yeast Vam3p. If true, this would imply that one syntaxin, PepA Pep12, is regulated at multiple levels by different SM proteins. In this paper we establish that PepA Pep12 exerts its syntaxin function across the whole endocytic pathway beyond sorting endosomes. We further
show that two SMs, Vps45 and Vps33, regulate $\text{Pea}^{\text{Pep12}}$, with Vps33 acting in the context of two MTCs.

**Results**

The biogenesis of endocytic compartments occurs by maturation, which involves lipid and protein sorting events facilitated by the balance between anterograde and retrograde traffic derived from and destined to the Golgi, respectively, by the invagination of portions of endosome membranes and associated proteins to form multivesicular bodies (MVBs), and by homotypic membrane fusion events. This dynamic process complicates the definition of the different stages. Here we divide the *A. nidulans* endocytic pathway into four stages: post-Golgi endosomes (PGEs), EEs, LEs and vacuoles.

EEs are distinguishable by their rapid movement using MT motors (Lenz et al., 2006, Abenza et al., 2009, Zekert & Fischer, 2009, Seidel et al., 2013) and by their content of Rab$^{\text{RAB5}}$, which recruits to EEs both the CORVET tethering complex and the Vps34 kinase (Abenza et al., 2010). Vps34 synthesizes PtdIns3P, a key landmark that engages the MVB pathway machinery (Henne et al., 2013). Upstream of EEs there is a loosely defined sorting endosome that receives both membrane and proteins that escape the TGN without being orderly sorted into post-Golgi carriers as well as endocytic traffic (Holthuis et al., 1998b, Lewis et al., 2000, Prescianotto-Baschong & Riezman, 2002). It has been denoted the post-Golgi endosome (PGE) (Hettema et al., 2003) to underline its dual personality, which is ‘resolved’ by segregation into different membrane domains bound to the TGN or leading to EEs. In yeast, the PGE involves the SNAREs Tlg1p and Tlg2p and is unaffected by pep12Δ. The transition from PGEs to EEs is mediated by RAB5s (Prescianotto-Baschong & Riezman, 2002). The transition of EEs into LEs is determined by the substitution of RAB5 by RAB7 (Rab$^{\text{RAB5/Vps21}}$ and Rab$^{\text{RAB7/Ypt7}}$ in *A. nidulans* and *S. cerevisiae*, respectively) (Balderhaar et al., 2013, Epp et al., 2011, Nordmann et al., 2010, Kummel & Ungermann, 2014, Rink et al., 2005, Poteryaev et al., 2010, Markgraf et al., 2009, Peplowska et al., 2007, Abenza et al., 2010, Abenza et al., 2012). LEs undergo fusion among them and with vacuoles, in a process that mediated by HOPS, a Rab$^{\text{RAB7/Ypt7 effector.}}$
Robust localization of PepA\textsuperscript{Pep12} to endovacuolar membranes

Fig. S1 shows a three-way alignment of *S. cerevisiae* Vam3p and Pep12p with their *A. nidulans* relative PepA\textsuperscript{Pep12} (AN4416) (Findon et al., 2010). Phylogenetic analysis indicates that AN4416 is closer to Pep12p, but PepA\textsuperscript{Pep12} shares features of both Pep12p and Vam3p, in agreement with the prediction that PepA\textsuperscript{Pep12} plays the roles of both. Consistent with its playing roles at multiple levels of the endocytic pathway, PepA\textsuperscript{Pep12} displays ‘broad’ localization across the whole endovacuolar system, co-localizing with RabA\textsuperscript{RAB5} on EEs and with RabS\textsuperscript{RAB7} on LEs and vacuoles (Abenza et al., 2012).

The localization of type-II single-pass membrane proteins such as PepA\textsuperscript{Pep12} is influenced by the length and composition of the transmembrane domain (TMD) (Sharpe *et al.*, 2010). PepA\textsuperscript{Pep12} and *S. cerevisiae* Pep12p display notable differences in their TMDs (Fig. 1A). For example, whereas the TMD of the yeast SNARE contains 18 residues (Reggiori *et al.*, 2000), that of PepA\textsuperscript{Pep12} contains 20-21. Moreover, the TMDs of filamentous fungal Pep12s often display at their cytosolic border two Cys residues (Cys253 and Cys254 in PepA\textsuperscript{Pep12}) that are potentially lipidable (Fig. 1A), which would further increase its hydrophobicity. We challenged the robustness of PepA\textsuperscript{Pep12} localization with missense mutations aimed at diminishing the hydrophobicity of the TMD (Fig. 1A), using an ectopic, pyroA-targeted GFP-PepA\textsuperscript{Pep12} expression construct driven by the *gpdA\textsuperscript{mini}* promoter (Pantazopoulou & Peñalva, 2009), such that the mutants also contained endogenous pepA (PepA\textsuperscript{Pep12} is severely debilitating and thus mutations delocalizing this SNARE were expected to impair growth markedly). Western blots showed that the ectopic construct results in overexpression of PepA\textsuperscript{Pep12} (Fig. 1B; anti-PepA\textsuperscript{Pep12} western blot, compare GFP-tagged and endogenous wild-type PepA\textsuperscript{Pep12}). Despite overexpression, the localization pattern of GFP-PepA\textsuperscript{Pep12} expressed under the control of the *gpdA\textsuperscript{mini}* promoter closely reflects that of the endogenously tagged version (Findon *et al.*, 2010)(the latter is shown for reference in Fig. S2 and Movie S1). Figs. 1B-E, and S2 show that irrespectively of its levels of expression wild-type PepA\textsuperscript{Pep12} localizes to spherical vacuoles, static puncta representing LEs and motile EEs (Abenza et al., 2012).

A double Cys253Ser/Cys254Ser substitution did not perturb this pattern (Fig. 1C, D and E), nor did it affect the steady-state levels of the protein (Fig. 1B; note that residue
substitutions cause minor shifts in the motility of the protein in SDS-polyacrylamide gels). However, the mutation augmented the number of puncta in the tip regions, which are largely devoid of vacuoles (Fig. 1C). The number of puncta per hyphal length unit in the tip regions of n=11 mutant and n=12 wild-type hyphae was moderately yet significantly higher in the mutant (1.3 +/- 0.06 S.E. vs. 1.0 +/- 0.04 S.E. puncta/micron; p=0.0002). This increase appears, at least in part, attributable to EEs, as mutant tips showed, on average, 0.95 +/- 0.07 SE moving trajectories per micron; n=12 kymographs), a value that was significantly higher (p = 0.027) than the wild-type count (0.65 +/- 0.11 SE; n = 8 kymographs). Thus, the loss of potentially lipidable Cys residues affects the steady state PepA\textsuperscript{Pep12} localization, but the resulting changes are very minor, indicating that these residues alone do not play a critical role.

We next challenged the hydrophobicity of the TMD. In yeast Pep12p, substitution by Asp of an Ile residue in position 5 of the TMD results in the sorting of the SNARE to the vacuolar lumen \textit{via} the MVB pathway (Reggiori et al., 2000). The equivalent Ile257Asp substitution in PepA\textsuperscript{Pep12} did not cause this effect, and consequently the levels of protein were normal (Fig. 1B and C). However, it affected the localization of the protein to a greater extent than Cys253Ser/Cys254Ser. PepA\textsuperscript{Pep12} Ile257Asp, although still localizing to endocytic compartments, was partially shifted to ER membranes. ER labeling was clearly noticeable by the GFP fluorescence of the nuclear membranes (Figs. 1C and S3), even though the mutant protein was also detectable in peripheral ER strands (Fig. S3). Therefore, equivalent substitutions in Pep12 orthologues result in markedly different effects, with those of PepA\textsuperscript{Pep12} Ile257Asp being relatively modest. However, when Ile257Asp was combined with the cysteine substitutions, fluorescence was very weak and completely displaced to the cytosol (Fig. 1, C and D), reflecting that this mutant protein was degraded (Fig. 1B; a faint proteolysis band was detected by anti-GFP blotting). Thus, the hydrophobicity of the TMD cooperates with potentially lipidable Cys residues to ensure a very robust localization of PepA\textsuperscript{Pep12} to the endovacuolar system.

Only a very minor proportion of TlgB\textsuperscript{Tlg2} localizes to EEs
In previous work we established that GFP-TlgB\textsuperscript{Tlg2} colocalizes with the TGN cisternae marker mRFP-PH\textsuperscript{OSBP} (Pinar et al., 2013). \textit{A. nidulans} TGN cisternae are seen as puncta that do not move rapidly and that are generally larger than EEs (Pinar et al., 2013) (Fig.
However, others have detected *A. nidulans* TlgB^{Tlg2} on moving structures (Kuratsu *et al.*, 2007, Zekert & Fischer, 2009). We revisited our localization studies, trying to detect GFP-TlgB^{Tlg2} on moving EEs by acquiring time stacks with high time resolution to track rapid particle movement with kymographs. To increase the chance of detecting TlgB^{Tlg2} on EEs avoided the potential interference caused by the abundant, yet markedly polarized TGN puncta (Kuratsu *et al.*, 2007, Zekert & Fischer, 2009). We indeed detected a few motile structures resembling EEs by focusing our observations on regions distant from the tip. We demonstrated that these motile structures are endosomes by their colocalization with the EE marker RabA^{RAB5} (Fig. 2B) (Movie S2). All the GFP-TlgB^{Tlg2} trajectories co-localized with mCh-RabA, although not every mCh-RabA trajectory was labeled with TlgB^{Tlg2}. Therefore these data confirm that a vast majority of TlgB^{Tlg2} resides in the TGN, with only a minor proportion reaching EEs. However, we note that in all the above experiments GFP-TlgB^{Tlg2} was expressed under the control of an ectopic expression construct driven by the moderately strong *gpdA* {\textsuperscript{mini}} promoter (Pantazopoulou & Peñalva, 2009), and thus we cannot rule out that this minor proportion of EE-localized GFP-TlgB^{Tlg2} is an artifact resulting from overexpression. It is also worth noting that the above results do not negate the possibility that TlgB^{Tlg2} plays some role in the PGE (see below), as the almost exclusive localization of the protein to the TGN in the steady-state (Pinar *et al.*, 2015) might be explained if this syntaxin were very efficiently retrieved from endosomes back to the TGN and/or if we would not have been able to resolve TGN cisternae from PGEs by optical microscopy; for example if TGN cisternae and PGEs were very closely associated, or if PGEs were punctate structures markedly less abundant than TGN cisternae.

**Normal growth of the tlgBΔ mutant is incompatible with a direct involvement of TlgB^{Tlg2} in endosome biogenesis**

The biogenesis of RAB5 EEs and the ability of these EEs to mature into LEs are physiologically crucial processes for *A. nidulans* (Abenza *et al.*, 2010, Calcagno-Pizarelli *et al.*, 2011). Consequently, ablation of SNAREs involved in the biogenesis of EEs, or in the EE-to-LE transition is expected to be severely debilitating. Notably, *tlgBΔ* did not cause any growth defect under any condition tested (Fig. 3A). One possible explanation for the lack of effect of *tlgBΔ* is that the orthologue of the Qc-SNARE Tlg1 (Holthuis *et al.*, 1998a) substitutes for the syntaxin role of TlgB^{Tlg2}. We
deleted the gene encoding *A. nidulans* TlgA<sup>Tlg1</sup>. Like *tlgBΔ*, *tlgAΔ* mutants showed no growth defect either (Fig. 3A), nor did a double *tlgAΔ tlgBΔ* mutant, ruling out that functional redundancy could explain the lack of effect.

Thus genetic evidence strongly indicates that TlgB<sup>Tlg2</sup> is not involved in the maturation of EEs. Moreover, considering that TlgB<sup>Tlg2</sup> localizes to the TGN and that hyphal growth is crucially dependent on exocytosis and Golgi function (Pinar et al., 2013) the normal growth of the *tlgBΔ* mutant establishes that TlgB<sup>Tlg2</sup> is also dispensable for exocytosis, even when TlgA<sup>Tlg1</sup> is also absent. Taking into account that yeast Tlg1p and Tlg2p are involved in homotypic fusion events in the TGN (Brickner et al., 2001) and that *tlg1Δ tlg2Δ* mutants show slight morphological abnormalities (Holthuis et al., 1998a), the completely normal growth of the *tlgAΔ tlgBΔ* double mutant of *A. nidulans*, whose hyphae are exquisitely dependent on exocytosis to maintain polarity, was somewhat unexpected. Thus we considered the possibility that an extragenic mutation arising spontaneously during transformation were remediating a severe growth defect resulting from *tlgAΔ tlgBΔ*, resembling the situation that we described previously during knock-out studies of ESCRT genes (Calcagno-Pizarelli et al., 2011). This possibility was rejected using classical genetics, after demonstrating that the normally growing progeny of a cross between wild-type and *tlgAΔ tlgBΔ* parental strains contained *tlgAΔ tlgBΔ* progeny at the frequency expected if only the two knock-out alleles (*i.e.* without a modifier mutation in a third gene) were segregating in a Mendelian fashion (Fig. S4).

**TlgB<sup>Tlg2</sup> is involved in traffic between an endocytic compartment and the Golgi**

We obtained evidence that TlgB<sup>Tlg2</sup> and TlgA<sup>Tlg1</sup> act in a pathway connecting an endocytic compartment with the TGN by testing the effects that *tlgAΔ* and *tlgBΔ* cause in the localization of the exocytic R-SNARE SynA. SynA normally localizes to the SPK and to an apical crescent, reflecting its continuous delivery to the apex combined with its efficient endocytosis and retrieval from endocytic compartments to the TGN, from which it returns to the plasma membrane by exocytosis (Fig. 3B) (Taheri-Talesh et al., 2008, Abenza et al., 2009, Pantazopoulou & Peñalva, 2011, Pinar et al., 2013). Fig. 3B shows that the apical SynA crescent of single *tlgAΔ* and *tlgBΔ* mutants, and of the double *tlgAΔ tlgBΔ* mutant is abnormal, being consistently shorter than in the wild type. Moreover, *tlgAΔ* and, to a lesser extent, *tlgBΔ* reduced the overall signal of SynA in the
tip region (the most apical 5 µm), with the double *tlgAΔ tlgBΔ* mutant behaving as *tlgAΔ* (Fig. 3C). These observations suggest that part of SynA is diverted towards vacuolar degradation in the *tlgAΔ tlgBΔ* mutant, as would be expected if the mutation(s) impair(s) the recycling of SynA from the PM to the TGN via an endocytic compartment. This role of TlgA/TlgB in retrograde traffic is consistent with the synthetically lethal interaction between *rabCΔ* (ablating RabC RAB6, a master regulator of retrograde traffic) and *tlgBΔ* (Pantazopoulou & Peñalva, 2011). The endocytic compartment used by the recycling pool of SynA cannot be EEs, as *rabBΔ* (ablating RabB RAB5) does not affect SynA distribution, even though it affects EEs markedly (Abenza et al., 2010). By analogy to yeast, we propose that this endocytic compartment is the PGE.

*TlgB Tlg2* seems largely dispensable, suggesting that another t-SNARE efficiently compensates for its absence from the TGN, with the ‘other’ Golgi syntaxin, SedV Sed5, being the obvious candidate. Synthetic interactions between *tlgBΔ* and *sedVΔ* cannot be tested because the latter is lethal, but we have previously characterized a conditional *sedV1 ts* allele that behaves as hypomorph at permissive temperature (Pinar et al., 2013). We tested if this *ts* allele shows a synthetic interaction with *tlgBΔ* or *tlgAΔ* at a permissive temperature, after recovering progeny of heterozygous crosses at 30ºC (a temperature at which *sedV1* grows reasonably well). We were unable to recover *tlgBΔ sedV1*, or *tlgAΔ sedV1* combinations, even though other markers recombined freely, which strongly suggested that *tlgBΔ sedV1* and *tlgAΔ sedV1* double mutations are lethal (Table I). Indeed we deleted *tlgB* in a *sedV1* background by transformation, and demonstrated, by heterokaryon rescue, that this combination is lethal (Fig. S5). Thus TlgB<sup>Tlg2</sup> or TlgA<sup>Tlg1</sup> are dispensable only if SedV<sup>Sed5</sup> is normal, but become crucial if the latter is compromised. These results agree with the conclusion of Pelham and coworkers that the Golgi can function with a single syntaxin, SedV Sed5 (Holthuis et al., 1998a). Experiments described below indicate that one role of SedV<sup>Sed5</sup> besides that in intra-Golgi traffic is ‘accepting’ retrograde traffic from endosomes.

**Detection of the Aspergillus nidulans endovacuolar SNARE interactions**

To clarify the SNARE partners involved in the different endocytic stages, we characterized the spectrum of interactions by immunoprecipitation experiments, using antisera raised against PepA<sup>Pep12</sup>, TlgB<sup>Tlg2</sup> (TGN/endosome interface) and, as control,
Sed\textsuperscript{V\textsubscript{Sed}}\textsubscript{5} (Golgi). Using strains expressing GFP-tagged versions of these syntaxins we confirmed the specificity of each antiserum for immunoprecipitation (Fig. 4A). Next we incubated antisera with a panel of seven cell extracts expressing, from gene replacement alleles, one of the Qb-, Qc- and R-SNAREs potentially acting in the endocytic pathway, tagged with a triple hemagglutinin epitope (HA3). Immunoprecipitates were then analyzed by anti-HA western blotting. Western blot data are shown in Fig. 4B, whereas a graphical depiction is shown in Fig. 4C, in which three different sizes of blue circles are used to represent the approximate efficiency of co-immunoprecipitation of the different syntaxin-partner combinations (estimated visually as the relative signal of co-immunoprecipitated versus non-precipitated HA3-tagged SNARE prey present in the flow through), and red dots indicate no immunoprecipitation (i.e. a signal equal to that obtained when pre-immune serum was used).

Pep\textsubscript{A}\textsuperscript{Pep12} was the sole syntaxin associating with all preselected endocytic SNAREs. Of these, only Ykt6 could not be functionally tagged by gene replacement, indicating that addition of the HA3 epitope impedes its normal function. Nevertheless results for Ykt6 are included because this R-SNARE interacts preferentially with Pep\textsubscript{A}\textsuperscript{Pep12}, weakly with Sed\textsubscript{V}\textsuperscript{Sed5} and not at all with Tlg\textsubscript{B}\textsuperscript{Tlg2}, indicating that Ykt6-HA3 retains at least some specificity (Fig. 4B and C).

Tlg\textsubscript{B}\textsuperscript{Tlg2}, expected to act at the TGN/PGE interface is notable in that it interacts solely with the ‘broad domain’ Qb SNARE Vti1, with Tlg\textsubscript{A}\textsuperscript{Tlg1} as Qc and with SynA as R-SNARE (Fig. 4B and C), contrasting with the promiscuity of interactions displayed by Pep\textsubscript{A}\textsuperscript{Pep12}. As SynA recycles to the TGN from endocytic compartments, these data suggest that Tlg\textsubscript{B}\textsuperscript{Tlg2}, Tlg\textsubscript{A}\textsuperscript{Tlg1}, Vti1 and SynA form the SNARE bundle by which retrograde traffic departing from a ‘pre-RAB5’ PGE fuses with the TGN, as in \textit{S. cerevisiae} (Holthuis et al., 1998a, Lewis et al., 2000).

Acting at the opposite ‘end’ of the endocytic pathway, the \textit{S. cerevisiae} homotypic vacuolar fusion SNARE bundle involves Vam3p forming a complex with Vti1p (Qb), Vam7p (Qc) and the R-SNARE Nyv1p (Ungermann \textit{et al.}, 1999, Nichols \textit{et al.}, 1997). Notably, \textit{A. nidulans} Vam7 exclusively associates with Pep\textsubscript{A}\textsuperscript{Pep12}. Vti1 immunoprecipitated efficiently with Pep\textsubscript{A}\textsuperscript{Pep12} as well, and to a much lesser extent with Tlg\textsubscript{B}\textsuperscript{Tlg2} and Sed\textsubscript{V}\textsuperscript{Sed5}, and Nyv1 immunoprecipitated both with Pep\textsubscript{A}\textsuperscript{Pep12} and with
SedV\textsuperscript{Sed5} to a similar extent. Thus these data provide strong experimental support for the contention that in \textit{A. nidulans} PepA\textsuperscript{Pep12} takes over the role of Vam3p in yeast, associating with the same (equivalent) partners.

In agreement with the postulated multiplicity of roles for PepA\textsuperscript{Pep12} across the endocytic pathway, PepA\textsuperscript{Pep12} pulls down two other Qc SNAREs, TlgA\textsuperscript{Tlg1} and Syn8, besides Vam7 (Fig. 4B and C). As Vam7 acts at vacuole-proximal steps, TlgA\textsuperscript{Tlg1} and Syn8 must play roles at earlier endocytic compartments. Unlike Syn8, TlgA\textsuperscript{Tlg1} does not engage TlgB\textsuperscript{Tlg2}, suggesting that the Syn8/PepA\textsuperscript{Pep12} combination is further away from the endocytic internalization step than the TlgA\textsuperscript{Tlg1}/TlgB\textsuperscript{Tlg2} combination.

Data discussed above suggest that alternative yet functionally efficient SNARE combinations must ensure that retrograde traffic arrives at the Golgi in the absence of TlgB\textsuperscript{Tlg2} and TlgA\textsuperscript{Tlg1}. The pattern of SNAREs co-immunoprecipitating with SedV\textsuperscript{Sed5} (Figure 4B and C) strongly suggested that these alternative pathway(s) involve the Golgi syntaxin SedV\textsuperscript{Sed5}, which associates with TlgA\textsuperscript{Tlg1}, Vti1 and SynA, \textit{i.e.} with the same Qb, Qc and R-SNAREs as TlgB\textsuperscript{Tlg2}, supporting the possibility that compositionally similar retrograde SNARE complexes involving each of the two Golgi syntaxins exist. Of note, Syn8 associates with SedV\textsuperscript{Sed5} with similar efficiency as TlgA\textsuperscript{Tlg1} (Fig. 4B and C). Functional overlap between Syn8 and TlgA\textsuperscript{Tlg1} would explain the inability of \textit{tlg4A} to block retrograde traffic completely (Fig. 3). In yeast, Tlg1p and Syn8p are interchangeable, providing similar Pep12p-dependent function, but neither appears to associate with Sed5p (Lewis & Pelham, 2002), in contrast with \textit{A. nidulans} Syn8. As a further example of the promiscuity of SedV\textsuperscript{Sed5}, this syntaxin associates with the vacuolar R-SNARE Nyv1 (Fig. 4B).

In summary, data are consistent with the existence of two SedV\textsuperscript{Sed5}-mediated retrograde pathways, both involving the multifunctional Qb SNARE Vti1: One implicating TlgA\textsuperscript{Tlg1} and SynA (or Ykt6), perhaps departing from more TGN-proximal endosomes; And another departing from vacuole-proximal endosomes, involving Syn8 and one of three possible R-SNAREs (Fig. 4C).
The severe growth impairment of the \textit{vps45}\textDelta\ mutant strongly indicates that Vps45 regulates PepA\textsuperscript{Pep12}.

Taken together the above experiments strongly indicate that PepA\textsuperscript{Pep12} regulates all fusion steps between EEs and the vacuole downstream of the TlgB\textsuperscript{Tlg2} PGE. Such a role is expected to be important for normal growth, for which endosome maturation is crucial (Calcagno-Pizarelli et al., 2011, Abenza et al., 2010). Indeed, in sheer contrast with \textit{tlgB}\textDelta, \textit{pepA}\textDelta is very severely debilitating, as expected for a mutation ablating a syntaxin mediating the biogenesis of EEs (Fig. 5A).

Sec1/Munc-18 (SM) proteins such as Vps45 regulate the activity of SNAREs (Sudhof & Rothman, 2009, Jahn & Fasshauer, 2012). The principal SNARE binder of yeast Vps45p is Tlg2p (Paumet et al., 2001, Dulubova et al., 2002, Nichols et al., 1998), but genetic evidence suggests that Vps45p has additional targets besides Tlg2p (Nichols et al., 1998), possibly Pep12p (Burd et al., 1997). In \textit{A. nidulans}, Vps45 is recruited to EEs by Rab\textsuperscript{RAB5} (Abenza et al., 2010). Notably, whereas \textit{tlgB}\textDelta has no effect on growth (Fig. 3), \textit{vps45}\textDelta is severely debilitating (Abenza et al., 2010) (Fig. 5A). Thus the important physiological role of \textit{A. nidulans} Vps45 cannot be regulating TlgB\textsuperscript{Tlg2} SNARE bundles. Fig. 5A shows that indeed the severe growth defect of the \textit{A. nidulans} \textit{pepA}\textDelta mutant is indistinguishable from that resulting from \textit{vps45}\textDelta, and strikingly different from that of \textit{tlgB}\textDelta, strongly indicating that the SM protein Vps45 crucially regulates PepA\textsuperscript{Pep12}. This contention implies that that \textit{pepA}\textDelta and \textit{vps45}\textDelta should not show additivity, which could not be tested because the severe growth phenotype of the single mutants precluded construction of a \textit{pepA}\textDelta \textit{vps45}\textDelta double mutant.

SltA is a transcription factor regulating cation homeostasis (Spielvogel \textit{et al.}, 2008, Findon \textit{et al.}, 2010). Loss-of-function mutations in \textit{sltA} rescue the otherwise severely debilitating ablation of components of the ESCRT complexes, which are essential for endosome maturation (Calcagno-Pizarelli et al., 2011). Fig. 5B shows that the severe \textit{vps45}\textDelta growth defect is markedly reduced by \textit{sltA}\Delta, consistent with the conclusion that the critical role of Vps45 is on EE maturation. Notably, the \textit{pepA}\textDelta growth defect is also remediable by \textit{sltA}\Delta, although to a much lesser extent (Fig. 5B). Our interpretation for these differences in suppression is that PepA\textsuperscript{Pep12} is indeed the only syntaxin acting
between EEs and the vacuole and no other syntaxin that can efficiently substitute its role, whereas the role of Vps45 can be, at least in part, fulfilled by Vps33 (see below).

**PepA**<sub>Pep12</sub> interacts with the SM proteins Vps45 and Vps33

The genetic data described above indicate that Vps45 regulates PepA<sub>Pep12</sub>. In addition, PepA<sub>Pep12</sub> must be regulated by another SM protein, Vps33 (Subramanian et al., 2004, Lobingier & Merz, 2012). Physiologically Vps33 acts in the context of two multisubunit tethering complexes, CORVET and HOPS, which mediate maturation of EEs into LEs and vacuoles. CORVET and HOPS are effectors of the endocytic pathway RABs, Rab5 and Rab7 (denoted RabB<sup>RAB5</sup> and RabS<sup>RAB7</sup> in *A. nidulans*, respectively) (Peplowska et al., 2007, Brocker et al., 2012, Nordmann et al., 2010, Abenza et al., 2010, Abenza et al., 2012). Thus we tested the SM proteins interacting with PepA<sub>Pep12</sub> and TlgB<sub>Tlg2</sub> by immunoprecipitation, using cell extracts expressing HA3-tagged versions of Vps45 and Vps33 (from the respective endogenously tagged genes). In these assays Vps45 co-purified efficiently with TlgB<sub>Tlg2</sub> as in yeast (Nichols et al., 1998) whereas Vps33 did not (Fig. 6). In contrast, both Vps45 and Vps33 co-purified with PepA<sub>Pep12</sub>, strongly supporting the contention that PepA<sub>Pep12</sub> is regulated by these two SMs.

In late endosomes/vacuoles Vps33 in HOPS binds directly the SNARE domains of Vam3p, Vam7p and Nyv1p (Lobingier & Merz, 2012). It could be argued that Vps33 binding to PepA<sub>Pep12</sub> is indirect (for example mediated by the other SNAREs associating with PepA<sub>Pep12</sub>). To determine whether the Vps33-PepA<sub>Pep12</sub> interaction is direct and in view of our inability to produce in *E. coli* the complete cytosolic domain of TlgB<sub>Tlg2</sub> in a soluble manner, we used a transcription-and-translation (TNT) coupled reticulocyte lysate system to synthesize soluble cytoplasmic domains of TlgB<sub>Tlg2</sub> and PepA<sub>Pep12</sub>, as well as HA3-tagged full-length versions of Vps33 and Vps45. Next we mixed different syntaxin/SM combinations, immunoprecipitated the mixtures with anti-syntaxin antisera and monitored SM co-purification by western blotting (Fig. 6). These assays established that the Vps45-TlgB<sub>Tlg2</sub> interaction, and the interactions of PepA<sub>Pep12</sub> with Vps33 and Vps45 are direct and thus, in the case of Vps33, interaction with PepA<sub>Pep12</sub> can take place in the absence of other CORVET or HOPS components.
**PepA\textsuperscript{Pep12} associates with CORVET and HOPS**

As noted above, yeast Vps33p binding to Vam3p is complex. Vps33 binds directly to the vacuolar SNARE-pin, possibly in its capacity to clasp SNARE complexes (Sudhof & Rothman, 2009). In addition, the core components of HOPS directly bind the syntaxin, thus recruiting Vps33p within the holo-complex to Vam3p (Lobingier & Merz, 2012). Therefore, to demonstrate further that *A. nidulans* PepA\textsuperscript{Pep12} takes over the roles of yeast Vam3p, we addressed whether PepA\textsuperscript{Pep12} associates with both CORVET and HOPS *in vivo*. To detect PepA\textsuperscript{Pep12}-HOPS and PepA\textsuperscript{Pep12}-CORVET interactions we used endogenously HA3-tagged Vps8 and Vps41 as CORVET- and HOPS-specific reporter, respectively. These proteins can only associate with SNAREs indirectly, as components of the interacting complexes. Fig. 7 shows that PepA\textsuperscript{Pep12} associates *in vivo* with both Vps8 (CORVET, EEs) and with its HOPS-specific equivalent Vps41 (LEs-vacuoles), further demonstrating that PepA\textsuperscript{Pep12} has the capacity to operate at every stage between EEs and vacuoles.

**Discussion**

**The domain of action of PepA\textsuperscript{Pep12}:** *A. nidulans* lacks a homologue of the *S. cerevisiae* vacuolar syntaxin Vam3p. The subcellular localization of PepA\textsuperscript{Pep12} to EEs, LEs and vacuoles strongly suggests that PepA\textsuperscript{Pep12} assumes the role of Vam3p. Pull-down experiments revealed that PepA\textsuperscript{Pep12} is capable of forming at least three SNARE bundles of different composition. One of these bundles contains the equivalents of the Qb-, Qc- and R-SNAREs that associate with *S. cerevisiae* Vam3p during vacuolar fusion. Thus PepA\textsuperscript{Pep12} is the only syntaxin acting in the endocytic pathway downstream of TlgB\textsuperscript{Tlg2}.

*S. cerevisiae* Pep12p continuously cycles between the Golgi and the endosomes, but in the steady state it localizes to endosomes because anterograde transport is faster than its retrieval back to the Golgi (Hettema et al., 2003, Black & Pelham, 2000). Gga1p/Gga2p adaptors sort Pep12p into endosome-bound clathrin carriers departing from the TGN (Black & Pelham, 2000). Sorting involves a FSDSPEFQ motif in Pep12p that is missing in Vam3p, which uses instead a direct AP-3-mediated pathway between the Golgi and the vacuole (Darsow *et al.*, 1998). However, a similar motif is present in *A. nidulans* (*YHDDPEFQ*), suggesting that PepA\textsuperscript{Pep12} uses the GGA pathway. Yeast Pep12p does
not normally reach the vacuole because it is efficiently retrieved from endosomes and dispatched to the Golgi in a Grd19/retromer coat-dependent manner (Hettema et al., 2003). Thus a simple mechanism by which PepA<sup>Pep12</sup> may have acquired the capability of invading vacuolar territory is by losing the capacity to be sorted in retromer carriers. Even though the sequence/structure code mediating Pep12p sorting by retromer is complex, a F6L I71P double substitution impedes it (Hettema et al., 2003). Neither Pep12p Phe6 nor Ile71 are conserved in PepA<sup>Pep12</sup>, in agreement with the above explanation.

The pathways connecting endosomes with the Golgi: An overall view of these pathways emerged from a systematic analysis of the ‘endosomal’ SNAREs associating with PepA<sup>Pep12</sup> and with each of the two Golgi syntaxins, SedV<sup>Sed5</sup> and TlgB<sup>Tlg2</sup>. Fig. 8A displays endosomal SNARE complexes represented as a ‘Pelham’s scheme’ (Pelham, H.G.R., personal communication), a type of representation in which SNAREs depicted at the same level have been found to interact with each other.

Our data are consistent with TlgB<sup>Tlg2</sup> acting at a very early stage of endocytosis: Lipids and proteins that are not orderly sorted into carriers at the TGN escape the boundaries of the Golgi and build up a compartment that receives endocytic traffic from the plasma membrane. As such this would be the ‘earliest endosome’ but given that these membranes share endocytic and Golgi identity it has been denoted ‘Post-Golgi endosome’ (Hettema et al., 2003)(PGE in Fig. 8B). The PGE is connected with the TGN by a retrograde pathway that retrieves fugitives back to the TGN and that is also used by proteins undergoing endocytic recycling, such as the R-SNARE SynA and, in all likelihood, the Dnf<sub>A</sub>Dnf<sub>1</sub> flippase (not tested here), to traffic between the PM and the TGN (Taheri-Talesh et al., 2008, Pantazopoulou & Peñalva, 2011, Schultzhaus et al., 2015, Valdez-Taubas & Pelham, 2003) (Fig. 8B). In addition, the PGE might recycle membranes containing SynA directly to the plasma membrane, through a pathway involving RecyA (Herrero et al., 2014), whose <i>S. cerevisiae</i> Recy1p homologue is an effector of the Ypt31p/Ypt32p RAB GTPases (Chen et al., 2005), the orthologues of the <i>A. nidulans</i> exocytic RabE<sup>RAB11</sup> (Pantazopoulou et al., 2014).

<i>A. nidulans</i> traffic connecting the PGE with the TGN must normally exploit TlgB<sup>Tlg2</sup> and its partner SNAREs TlgA<sup>Tlg1</sup>, Vti1 and SynA (Fig. 8A), as in <i>S. cerevisiae</i>
tlgAΔ and tlgBΔ result in a weak SynA distribution phenotype, possibly because other syntaxins/pathways departing from endosome compartments downstream of the PGE mediate retrograde transport to the Golgi (Bonifacino & Rojas, 2006) (see Fig. 8), and these are exploited by cargo that normally uses the TlgB
\[\text{Tlg2}\] pathway if this syntaxin is inactivated. The fact that tlgAΔ tlgBΔ does not affect growth in spite of the effect that it causes in SynA distribution suggests that the sum of the amounts of SynA reaching the apical dome biosynthetically and by alternative recycling pathways suffices to maintain normal apical extension.

Our data hint at the pathways that may be used if TlgB
\[\text{Tlg2}\] is ablated. PepA
\[\text{Pep12}\] associates in vivo with TlgA
\[\text{Tlg1}\], Vti1 and SynA (Fig. 8B), thus it might provide a platform for the landing of endocytic vesicles containing SynA in case of need. However, retrograde traffic to the Golgi must use SedV
\[\text{Sed5}\] instead, which forms complexes in vivo with appropriate Qb (Vti1), Qc (TlgA
\[\text{Tlg1}\] or Syn8) and R (SynA or Ykt6) specificity (Fig. 8A) (intra-Golgi complexes formed by SedV
\[\text{Sed5}\] have not been investigated here). In support for such a retrograde role of these SedV
\[\text{Sed5}\] bundle(s), S. cerevisiae Vti1 was implicated in traffic from the endosomes to the Golgi early on (von Mollard et al., 1997), Sed5p associates with Vti1p in vivo (Lupashin et al., 1997) and indeed Sed5p can form quaternary complexes in vitro with Vti1, Tlg1 and Ykt6 (Tsui et al., 2001). That SedV
\[\text{Sed5}\] can play TlgB
\[\text{Tlg2}\] roles is consistent with the finding that tlgBΔ becomes essential if SedV
\[\text{Sed5}\] is debilitated, which augments the previous observation with S. cerevisiae demonstrating that the Golgi can function with a single syntaxin, Sed5p (Holthuis et al., 1998a).

Rab6 homologues (RabC
\[\text{RAB6}\] in A. nidulans) are master regulators of retrograde traffic. The double tlgBΔ rabCΔ mutant is barely viable (Pantazopoulou & Peñalva, 2011), whereas rabCΔ strains grow reasonably well and tlgBΔ strains are wild-type. Thus, formally, RabC
\[\text{RAB6}\] plays TlgB
\[\text{Tlg2}\] -independent roles that, we propose, are mediated by cooperation with SedV
\[\text{Sed5}\]. Indeed RabC
\[\text{RAB6}\] colocalizes with SedV
\[\text{Sed5}\] substantially, and without RabC
\[\text{RAB6}\] the early Golgi is as fragmented (Pantazopoulou & Peñalva, 2011).

PepA
\[\text{Pep12}\] and the different stages of the endocytic pathway: The earliest stage of the endocytic pathway at which PepA
\[\text{Pep12}\] acts is the EE (Fig. 8B). TlgB
\[\text{Tlg2}\] reaches EEs
only occasionally, and thus the sharp $\text{TlgB}^{\text{Tlg2}}-\text{PepA}^{\text{Pep12}}$ boundary marks the border separating the mixed identity PGE and the fully committed EE, possibly because only the latter contains PtdIns3P. In contrast, EEs and LEs form a continuum of maturing membrane-bound compartments corresponding to the yeast pre-vacuolar compartment (PVC) (Fig. 8B) (Bowers & Stevens, 2005). These endosomes bidirectionally exchange traffic with the Golgi and undergo inward budding of vesicles to acquire multi-vesicular appearance. In the Fig. 8B model we propose that retrograde traffic departing from the PVC to the Golgi utilizes $\text{SedV}^{\text{Sed5}}$ as t-SNARE. At the vacuole, the end of the endocytic pathway, $\text{PepA}^{\text{Pep12}}$ associates with the orthologues of the known $S. \text{cerevisiae Vam3p}$ partners $\text{Vti1, Vam7 and Nyv1}$ (Fig. 8), demonstrating that it has the capability to functionally substitute for Vam3p.

The transition between the PGE and EEs is governed by $\text{RabB}^{\text{RAB5}}$, which determines the EE ‘identity barcode’. It recruits the SM protein Vps45 (Abenza et al., 2010). Vps45 almost certainly regulates $\text{PepA}^{\text{Pep12}}$ in fusogenic events involving incoming traffic from the Golgi (this work). $\text{RabB}^{\text{RAB5}}$ also recruits the CORVET complex, which acts in two ways: as tether, with Vps8 involvement (Markgraf et al., 2009), and via regulation by Vps33 of $\text{PepA}^{\text{Pep12}}$-mediated fusion. After a certain degree of maturation $\text{RabS}^{\text{RAB7}}$ substitutes for $\text{RabB}^{\text{RAB5}}$, HOPS substitutes for CORVET, and late endocytic compartments undergo fusion among themselves and with vacuoles, in a process that involves $\text{PepA}^{\text{Pep12}}$ regulation by Vps33, but now in the context of HOPS. Therefore $\text{PepA}^{\text{Pep12}}$ is regulated by two different SMs, Vps45 and Vps33, at three different steps, yet the mechanisms by which different SMs act at the right step are not known. We speculate that these mechanisms involve the ability of SM proteins to bind SNARE bundles (Carpp et al., 2006, Carr & Rizo, 2010) and to serve as template for SNARE assembly (Baker et al., 2015).
Experimental Procedures

Aspergillus strains, media and molecular genetics:

Aspergillus complete (MCA) or synthetic complete (SC) medium containing 1% glucose and 5 mM ammonium tartrate (i.e. 10 mM NH₄⁺) as carbon and nitrogen source, respectively, were used for growth tests and strain maintenance (Cove, 1966). Strains, which carried markers in standard use, are listed in Supplemental Table 1. Cassette for mutagenic gene replacement, including gene deletions and N- or C-terminal GFP or triple hemagglutinin epitope (HA3) tagging were assembled by fusion PCR, using *A. fumigatus* pyrG (pyrG<sup>af</sup>) as selective marker (Szewczyk *et al.*, 2006). MAD1739 (Supplemental Table 1), carrying a pyrG89 mutation resulting in pyrimidine auxotrophy and nkuAΔ mutation (Nayak *et al.*, 2005) to prevent non-homologous recombination was used as recipient strain for transformation, unless indicated otherwise. Deletion of *pepA*, *tlgB*, *tlgA* and *vps45* was carried out as detailed in Fig. S6. The correct integration of the transforming fragments was verified by diagnostic PCR.

Plasmids for *E. coli* and TNT expression

All expression vectors were derivatives of a Novagen’s pET21b, custom-modified to express polypeptides C-terminally tagged with deca-His, with or without HA3. Briefly, for rabbit immunization, p2184 encodes PepA<sup>Pep12</sup>(1-252)-His10; p2165 encodes TlgB<sup>Tlg2</sup>(1-191)-His10 and p2147 encodes SedV<sup>Sed5</sup>(1-320)-His10. For TNT expression, p2145 encodes TlgB<sup>Tlg2</sup> (1-312)-His10, whereas p2185 and p2144 encode full length Vps45-HA3 and Vps33-HA3, respectively (p2184 was used for TNT expression of PepA<sup>Pep12</sup>.

Rabbit polyclonal antisera against PepA<sup>Pep12</sup>, TlgB<sup>Tlg2</sup> and SedV<sup>Sed5</sup>

Rabbits were immunized with polypeptides containing cytosolic domains of the syntaxins corresponding to the amino acid residues indicated above. TlgB<sup>Tlg2</sup> 1-191 containing the Habc domain was the only cytosolic region that we were able to express in *E. coli* in high levels. The ORFs were PCR-amplified from cDNA and inserted into a vector derived from the Novagen pET series derivative to obtain syntaxin polypeptides with a C-terminal His<sub>10</sub> tag. The resulting plasmids were introduced into the *E. coli* strain BL21 carrying the compatible plasmid pRIL (Stratagene), which is a derivative of pACYC184 encoding *E. coli* tRNA-genes for the 'bacterially infrequent' Arg, Ile and
Leu codons AGG, AGA, AUA and CUA. Expression was induced for 4 h at 37°C with 0.1 mM isopropyl-β-D-thiogalactopyranoside. Proteins were purified from cleared lysates by incubation, at 4°C, with 0.5 ml of Ni-Sepharose 6 Fast Flow beads (GE Healthcare). Beads were washed repeatedly with phosphate buffer saline (PBS) containing 75 mM imidazole followed by PBS with 100 mM imidazole before proteins were eluted with 100 mM EDTA. Imidazol was removed by gel filtration, using pre-packed Sephadex G-25 columns (GE Healthcare) equilibrated with PBS. Protein material in the exclusion volume was lyophilized and used to immunize rabbits (by Davids Biotechnologie GmbH, Regensburg, Germany). For TlgB^{Tlg2::His}_{10} purification, 6 M urea was used in the bacterial lysis, washing and elution buffers to avoid protein aggregation. The elution buffer was PBS containing 1.5 M urea and 100 mM EDTA.

**Immunoprecipitation assays**

Immunoprecipitation assays were performed with 5 mg of *A. nidulans* protein extracts (Abenza et al., 2010) in protein extraction buffer containing 20 mM Tris-HCl pH 8, 110 mM KCl, 10% (vol/vol) glycerol, 1% (vol/vol) Triton X-100, 5 mM EDTA, 2 µM MG132 and Complete ULTRA Tablets of EDTA-free protease inhibitor cocktail (Roche). Extracts were mixed with 5 µl of each antiserum (or pre-immune serum for controls) in Pierce® Centrifuge Columns (Thermo). The mixtures were incubated for 2 h at 4°C in a rotating wheel before adding 40 µl of Protein A-Sepharose™ 4 Fast Flow (GE Healthcare). Columns were then further incubated for 2 h at 4°C in a rotating wheel before washing the beads three times (10 min at RT in a rotating wheel) with 0.5 ml of chilled protein extraction buffer (containing 110 mM, 500 mM and 750 mM KCl for each of the successive washes). Bound proteins were eluted in 40 µl of Laemmli sample buffer. 20 µl aliquots were resolved in 12% SDS-polyacrylamide gels and transferred to nitrocellulose for anti-HA Western blotting. Western blots were reacted with Roche's anti-HA rat mAb (1/1000) as primary antibody and with Southern Biotechnology (Birmingham, AL) peroxidase-coupled goat anti-rat IgM+IgG antiserum (1:4000) as secondary antibody. Proteins were detected using Amersham Biosciences ECL.

**Endosomal SNARE tagging**

Endosomal SNAREs were N- or C-terminally (when appropriate) HA-tagged by gene replacement as follows (note that underlined Met codons were removed from the constructs):
HA3-Vti1: (HA)₃-MSNPLTDAGSEMLSSYETE (…)
(HA3-Tlg1): (HA)₃-MDHADPFLQVQADVSLGLT (…)
(HA3-Syn8): (HA)₃-MPNPSQLFLLADHKLKLLE (…)
(Vam7): (HA)₃-APPLEISIIPTTTTSTTPP (…)
(HA3-SynA): (HA)₃-MSEQPYDYPYIPSAGSNAASGS (…)
(Ykt6-HA3): M₁(…)_QSKMFYTSAKKQNSCCIIM-(HA)₃
(Nyv1-HA3): M₁(…)_YLFVGMGCGLPAWGRCVG-(HA)₃

A schematic representation of the procedure in the case of N-terminal tagging is shown in Fig. S7. The SM proteins Vps33 and Vps45, and the CORVET/HOPS components Vps8 and Vps41 were C-terminally tagged with HA3 by gene replacement as follows:

(Vps8-HA3): M₁(…)_QHDGEWEMLVCVPVHVSE-(HA)₃ (Abenza et al., 2010)
(Vps41-HA3): M₁(…)_DGCRICALAKELEAVGDSEA-(HA)₃ (Abenza et al., 2012)
(Vps45-HA3): M₁(…)_GWPESEPSTAAGRLRNIGR-(HA)₃ (Abenza et al., 2010)
(Vps33-HA3): M₁(…)_NGDRMMDAALEKGFALTES-(HA)₃

Analysis of GFP fluorescence images

To quantify the relative abundance of GFP-SynA in hyphal tip regions (plasma membrane, cytosolic structures and SPK), we used sum projections of z-stacks (2 µm deep) of wild-type and mutant hyphae, acquired under the same conditions. These projections were used to determine integrated pixel intensity values of a rectangular ROI measuring 50 x 30 pixels (as 1 pixel = 0.103 µm, we defined arbitrarily the tip region as the most apical 5 µm of the hyphae, corresponding to the region where GFP-SynA largely predominates in the wild-type). Equivalent ‘empty’ regions in the images were used to determine background, which was subtracted before statistically analyzing the data with Prism 3.0.

To compare the number of punctate structures containing wt or Cys253,254Ser PepAPep12 we counted manually the number of these puncta in medial planes of hyphal tip regions (considering as such the approximately apex-proximal 25 µm regions of hyphal tip cells, usually devoid of vacuoles). Average figures per unit length were compared by unpaired t-test (Prism software). To compare the number of EE runs, time-
lapse sequences of hyphal tip regions as above were acquired every 150 msec (150 frames, 22 sec in total) and used to derive kymographs (Supplemental movie 1 shows an example of one such sequences). EE runs were tracked manually and average figures per tip cell and unit length in the mutant and the wt compared by unpaired t-test.

**In vitro protein-protein interactions**

Cytoplasmic domains of TlgB\textsuperscript{Tlg2} (residues 1-312) and PepA\textsuperscript{Pep12} (residues 1-252) and C-termini HA-tagged versions of Vps33 and Vps45 were independently produced using the TNT T7 Coupled Reticulocyte Lysate System (Promega). Briefly, 1 µg of each plasmid DNA was mixed with 25 µl of TNT\textsuperscript{R} rabbit reticulocyte lysate, 2 µl of TNT\textsuperscript{R} reaction buffer, 1 µl of TNT\textsuperscript{R} T7 RNA polymerase, 0.5 µl of 1 mM ‘- Leu’ amino acid mixture, 0.5 µl of 1 mM ‘- Met’ amino acid mixture, 1 µl of RNasin\textsuperscript{R} and nuclease-free water to a final volume of 50 µl, and incubated at 30°C for 90 minutes. 10 µl aliquots of the in vitro synthesized proteins were mixed in 500 µl of 110 mM KCl protein extraction buffer (see above) in Pierce\textsuperscript{R} Centrifuge Columns (Thermo) and incubated for 2 h at 4°C in a rotating wheel. Physical interactions were detected following immunoprecipitation and anti-HA immunoblotting as described above.

**Microscopy**

*A. nidulans* cells were cultured for 16-18 h in ‘watch minimal medium’ (WMM) (Peñalva, 2005) at 25-28°C using Lab-Tek chambers (Nalge Nunc International, Rochester, NY). Low level expression of mCherry-RabA driven by the *alcA*p was attained by using 0.05% fructose (w/v) as carbon source (non-inducing and non-repressing conditions) (Abenza et al., 2012). For all other experiments, 0.1% glucose (w/v) was used as sole carbon source. Mature endosomes/vacuoles were detected with CMAC as described (Abenza et al., 2009, Pantazopoulou & Peñalva, 2009). Images were acquired using a Hamamatsu ORCA ER-II camera (Hamamatsu, Hamamatsu, Japan) coupled to a Leica DMI6000B microscope (Leica, Wetzlar, Germany) driven by MetaMorph software (Molecular Dynamics, Sunnyvale, CA) and equipped with an EL6000 external light source for epifluorescence excitation. For localization experiments, simultaneous imaging of GFP and mCherry was carried out using a Dual-View imaging system (Photometrics, Tucson, AZ), using the recommended filter set (Pantazopoulou & Peñalva, 2009). MetaMorph software was used for contrast adjustment, Dual-View channel alignment, color combining, z-stack projections
(contrasted, when indicated, with the MetaMorph 'unsharp mask' filter) and for the assembly of kymographs from time-lapse series. When indicated, images were deconvolved using Huygens software (Scientific Volume Imaging, The Netherlands). Time-lapse sequences were converted to QuickTime using ImageJ (National Institutes of Health, Bethesda, MD).

**Phylogenetic analysis**

*A. nidulans* AN4416 (*PepAPep12*) and *S. cerevisiae* Pep12p and Vam3p protein sequences were aligned with CLUSTAL W (Thompson *et al.*, 1994) and inspected manually. A maximum likelihood tree was built from the alignment by PHYML version 4.0 using both parsimony and distance analysis (neighbour joining; NJ) with 1000 bootstrap replicates (Guindon & Gascuel, 2003). The tree was represented as a phylogram with Dendroscope.

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Table I. Inability to recover sedV\textsuperscript{ts} tlgB\textDelta and sedV\textsuperscript{ts} tlgA\textDelta double mutants in the progeny of heterozygous crosses

<table>
<thead>
<tr>
<th></th>
<th>n=80</th>
<th>tlgB\textsuperscript{+}</th>
<th>tlgB\textDelta</th>
<th>pyroA4</th>
<th>pabaA1</th>
<th>total</th>
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<td>sedV\textsuperscript{+}</td>
<td>14</td>
<td>28</td>
<td>24</td>
<td>19</td>
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<tr>
<td>sedV\textsuperscript{ts}</td>
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<td>0</td>
<td>18</td>
<td>19</td>
<td>38</td>
<td></td>
</tr>
</tbody>
</table>

Parental strains: sedV\textsuperscript{1}::pyrG\textsuperscript{89} pyrG89 pyroA4 nkuA::bar x wA4 tlgB\Delta::pyrG\textsuperscript{89} pabaA1 pyrG89

<table>
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<tr>
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<th>pantoB100</th>
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</thead>
<tbody>
<tr>
<td>sedV\textsuperscript{+}</td>
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<td>10</td>
<td>14</td>
<td>12</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>sedV\textsuperscript{ts}</td>
<td>27</td>
<td>0</td>
<td>15</td>
<td>11</td>
<td>27</td>
<td></td>
</tr>
</tbody>
</table>

Parental strains: pyrG89\textsuperscript{?}; sedV\textsuperscript{1}::pyrG\textsuperscript{89}; nkuA\textDelta::bar\textsuperscript{?}; pantoB100 x pyrG89\textsuperscript{?}; tlgA\Delta::pyrG\textsuperscript{89}; pyroA4 nkuA\textDelta::bar\textsuperscript{?}
Figure Legends

Figure 1. PepA^Pep12^ mislocalization due to substitutions in the TMD

(A) Multiple sequence alignment of the region encompassing the TMD and its environs in PepA^Pep12^ and fungal orthologues. Amino acid residues composing the *A. nidulans* PepA^Pep12^ TMD are boxed in red. The three residues that are affected by substitutions described in the text are indicated with arrows. Navy blue and ice blue indicate complete and partial conservation, respectively. See, Cal and Spo indicate *S. cerevisiae*, *Candida albicans* and *Schizosaccharomyces pombe* Pep12, respectively. The filamentous fungal proteins above are all PepA^Pep12^ orthologues. Ani, *A. nidulans*; Ang, *A. niger*; Fox, *Fusarium oxysporum*; Ncr, *Neurospora crassa*; Mor, *Magnaporthe oryzae*; Pbr, *Paracoccidioides brasiliensis*; Cim, *coccidioides immitis*; Bei, *Botrytis cinerea*. (B) Western blot analyses of strains carrying GFP-PepA^Pep12^ transgenes, with anti-PepA antiserum (left) or anti-GFP antibody (right). PepA indicates the endogenous protein. Actin was used as loading control for the anti-GFP blot. (C) Overall localization of wild-type GFP-PepA^Pep12^ and the indicated mutants. Images are maximal intensity projections of z-stacks. Note the GFP-PepA^Pep12^ staining of the nuclear envelopes in the Ile257Asp mutant (see also Fig. S3) (D) As in (C) but focusing on basal regions to visualize large vacuoles. (E) Kymographs derived from time-lapse series of wild-type and mutant GFP-PepA^Pep12^. Static structures giving rise to vertical lines are LEs or small vacuoles, whereas moving structures giving rise to diagonal lines are EEs.

Figure 2. Only a minor proportion of TlgB^Tlg2^ localizes to moving EEs

(A) A time-lapse sequence of a hyphal tip cell expressing GFP-TlgB^Tlg2^ was used to calculate kymographs across the indicated lines. Note that TGN cisternae predominate in the apex-proximal region but are also present elsewhere. Some structures showing rapid movement (arrowed) were detectable in apex-distal regions. (B) Moving TlgB^Tlg2^ structures colocalize with the EE marker mCherry-RabA. Kymographs were derived from Movie S2.

Figure 3. Phenotypic aspects of *tlg*Δ strains

(A) *tlgAΔ*, *tlgBΔ* and *tlgAΔ tlgBΔ* strains grow like the wild-type on the indicated media and temperatures. (B) Abnormal distribution of GFP-SynA in *tlgAΔ*, *tlgBΔ* and *tlgAΔ*
tlgBΔ strains. Images are central planes of deconvolved z-stacks. (C) Quantitation of total GFP-SynA fluorescence in a 50 x 30 pixel region encompassing the tip. Horizontal bars, mean. The differences between the WT and the tlgBΔ mutant are not statistically significant (n.s.) (Details in Experimental Procedures).

**Figure 4. Immunoprecipitation experiments with endosomal SNAREs**

(A) Specificity of antisera used for immunoprecipitation (IP). Extracts from strains expressing the indicated GFP-tagged syntaxins were immunoprecipitated with the indicated antisera and the precipitates were analyzed by anti-GFP western blotting. I, input (5%); IP, immunoprecipitate; FT, flow through (unbound). (B) A panel of cell-free extracts obtained from strains expressing HA3-tagged SNAREs predicted to act in the endocytic pathway were immunoprecipitated with the indicated anti-syntaxin antisera. ‘Pre’ indicates pre-immune serum, used as negative control. (C) Approximate efficiency of immunoprecipitation of the indicated SNAREs (columns) with each of the three anti-syntaxin antisera. Four circle sizes were used to denote different efficiencies. A small red circle indicates no immunoprecipitation detected. All IP experiments were performed at least three times with similar results. Data in (B) show pictures from representative experiments.

**Figure 5. Vps45 must crucially regulate PepA^{Pep12}**

(A) Growth phenotypes of the indicated strains at 37°C. (B) sltAΔ weakly suppresses vps45Δ: Different degrees of suppression by sltAΔ of the growth defects resulting from vps45Δ and pepAΔ. The indicated strains were streaked on plates that were incubated at 37°C.

**Figure 6. Interaction of the SM proteins Vps45 and Vps33 with PepA^{Pep12} and TlgB^{Tlg2}**

(A) Immunoprecipitation (IP) with indicated anti-syntaxin antisera, or with control pre-immune (pre) antisera of ‘prey’ cell extracts expressing HA3-tagged versions of Vps45 or Vps33, followed by anti-HA western blotting. In, input; PD, pulled-down (immunoprecipitated) material; FT, flow-through material. (B) Immunoprecipitation experiments as above but using in vitro-synthesized (with TNT) HA3-tagged Vps45 or Vps33 as preys and the cytosolic domains of PepA^{Pep12} and TlgB^{Tlg2} as baits. Orange and light green circles with a magenta dot depict the two SM proteins tagged with HA3.
Green and blue vertical bars indicate the two different syntaxins (Stx1 and Stx2), with only one being recognized, in each case, by specific antibodies (AB). Dotted circles indicate the possibility in (A) that a component of extracts bridges the interaction between the SM and the syntaxin. This possibility cannot take place in (B), as these experiments were made with proteins synthesized in vitro using coupled transcription-translation reactions driven by plasmid DNA templates.

**Figure 7. PepA\textsuperscript{Pep12} interacts with the CORVET or HOPS complexes**

The scheme on the top illustrates that maturation of EEs into LEs/vacuoles involves the progressive substitution of RAB5 and its effector CORVET by RAB7 and its effector HOPS. The two complexes share four core subunits (blue) and differ in two specific subunits (green in CORVET and pink in HOPS). Bottom: immunoprecipitation of extracts expressing HA3-tagged versions of Vps8 and Vps41 showing that both associate with PepA\textsuperscript{Pep12} in vivo.

**Figure 8. Model of endosomal SNARE bundles**

(A) Pelham’s schematic representation of the syntaxin-SNARE associations detected in this study. In this type of representation SNAREs that have been shown to interact are depicted at the same level, visually summarizing the promiscuity of some of the SNAREs. Note that any SNARE bundle is composed of one component each of the Qa, Qb, Qc and R groups. (B) A model of endosomal traffic (see text for details). Syntaxins acting at different steps are underlined. TlgB\textsuperscript{Tlg2} acts at three steps: at the TGN mediating intra-Golgi fusion (not depicted); At the PGE mediating fusion of incoming endocytic vesicles (blue line); and in the retrograde pathway connecting PGEs with the TGN. This pathway may also operate in those domains of EEs that have not yet acquired RAB5 (RAB5, magenta lollypops). PepA\textsuperscript{Pep12} acts at all other stages of endocytosis: EEs (containing RAB5), LEs (containing RAB7, black lollypops) and vacuoles (Vac). It mediates fusion events within the endocytic pathway (SNARE partners in black) and also the fusion of incoming vesicles derived from the TGN with RAB5-containing domains of EEs (where these are predominating) and LEs (where RAB7 predominates and RAB5 domains are minor). The apex-directed pathway departing from the TGN (grey) is used by SynA and DnfA\textsuperscript{Dnfl} to recycle to the PM with exocytic carriers. We propose that the Golgi can accept retrograde traffic from EEs and LEs using SedV\textsuperscript{Sed5} as syntaxin (dotted orange arrows), forming SNARE bundles with
the indicated Qa, Qb and Qc partners (orange lettering). Large yellow arrows indicate trafficking steps, which in endosomes and Golgi are mediated by maturation, rather than by vesicle traffic. A dotted line connecting the PGE to the PM reflects the hypothetical possibility (question mark) that this sorting endosome is able to deliver traffic to the PM, without passing through the TGN.

Supporting information

Figure S1
Three-way alignment of *A. nidulans* PepA<sub>Pep12</sub> with *S. cerevisiae* Pep12p and Vam3p. The GGA box of Pep12p, largely conserved in PepA<sub>Pep12</sub>, is boxed. The phylogram was obtained removing gaps from the alignment and with 1000 bootstrap replicates. The scale bar indicates the relative length of each branch. ClustalW was used for protein alignment.

Figure S2
Localization of endogenously tagged GFP-PepA<sub>Pep12</sub>. (A) Maximal intensity projection of a z-stack showing punctate structures of endosomes near the tip, larger puncta representing LEs and small vacuoles, and large vacuoles near the base. The linescan illustrates the tip-to-base increase in vacuole number and size. Single optical sections of the indicated regions were magnified to display better the morphology of the different endocytic compartments. (B) Kymograph analysis of a time series. The tracks of EEs are schematized with different colors on the right. Figures indicate the velocity of the EE in the corresponding section, with left- and right-pointing arrows indicating movement towards and away from the tip, respectively. (C) Example of GFP-PepA<sub>Pep12</sub> labeling of vacuoles near the basal conidiospore.

Figure S3
Ile257Asp shifts a proportion of GFP-PepA<sub>Pep12</sub> towards the ER. (A) Overall view of hyphae expressing wild-type or mutant Ile257Asp GFP-PepA<sub>Pep12</sub>. Blue arrows indicate nuclear envelopes; magenta arrowheads, vacuoles; green arrowheads, cortical ER. (B) Middle sections of wild-type and mutant hyphal tips annotated as in (A). (C) Subapical
regions of wild-type and mutant hyphae. All images were deconvolved with Huygens software.

**Figure S4**
The indicated double mutant *tlgAΔ tlgBΔ* strain was crossed with a wild type strain. Progeny were recovered on medium lacking pyrimidines, thus eliminating all *pyrG89* progeny. The remaining progeny, which in all cases showed wild-type growth, were genotyped by PCR as in the example shown. The table shows the expected and observed genotypic combinations with regard to the *tlgA* and *tlgB* loci. The two distributions are not significantly different by psi-square testing. Thus *tlgAΔ* and *tlgBΔ* display Mendelian behavior, ruling out the possibility that suppressor mutations in other genes were obscuring any growth impairment phenotype that might have resulted from the deletion alleles.

**Figure S5**
Double *sedV1 tlgBΔ* mutant strains are not viable. Top, colony phenotype of a *sedV1ts pyroA4* strain used as recipient of a transformation experiment with a *tlgBΔ:pyroA4f* DNA cassette (see scheme), compared to that of transformants selected for pyridoxine prototrophy. Note the sick and irregular aspect of the colonies in the latter. These were shown to be *tlgB+/tlgBΔ* heterokaryons by PCR genotyping (bottom), indicating that this combination of mutations is not viable.

**Figure S6**
Diagnostic products of PCR amplification reactions used to genotype gene knockouts. In all four cases, the relevant *A. nidulans* gene was replaced by *A. fumigatus pyrG*. PstI digestion was used to distinguish the otherwise similarly-sized PCR amplification bands of the *vps45Δ* mutant and the wild-type

**Figure S7**
Schematic representation of targeted gene replacements substituting endogenous SNARE coding regions by N-terminal HA-tagged versions

**Table S1**
Strains used in this work
Movie S1
Time-lapse sequence of a hyphal tip cell expressing endogenously tagged GFP-PepA\textsuperscript{Pep12}. Rapidly moving EEs and LEs/small vacuoles connected by tubular structures are visible. Frames taken every 0.15 sec. Time in sec:msec.

Movie S2
Time-lapse sequence of mCherry-RabA and GFP-TlgB\textsuperscript{Tlg2} accelerated 4 times. Frames taken every 0.25 sec. Time in sec:msec.