Genetic studies on the physiological role of CORVET in *Aspergillus nidulans*

by

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SUMMARY

CORVET and HOPS are protein complexes mediating the maturation of early endosomes (EEs) into late endosomes (LEs)/vacuoles. These hetero-hexamers share four 'core' components, Vps11, Vps16, Vps18 and Vps33, and differ in two specific subunits, CORVET Vps8 and Vps3 and HOPS Vps39 and Vps41. Whereas ablating HOPS-specific components has minor growth effects, ablating any CORVET constituent severely debilitates *Aspergillus nidulans* growth, buttressing previous work indicating that maturation of EEs into LEs is physiologically crucial. A genetic screen revealed that impairing the *slt* cation homeostasis pathway rescues the growth defect resulting from inactivation of the 'core' protein Vps33. Subsequent genetic analyses showed that the defect resulting from lack of any one of the five other CORVET components could similarly be rescued by *sltA*Δ eliminating the *slt* regulator SltA. Whereas double deletants lacking functionally non-equivalent components of the CORVET and HOPS complexes are rescued by *sltA*Δ, those lacking functionally equivalent components are not, suggesting that intermediate 'hybrid' complexes previously detected in yeast are physiologically relevant. *vps3*Δ, *vps8*Δ, *vps39*Δ and *vps41*Δ result in small vacuoles. This phenotype is remediable by *sltA*Δ in the case of CORVET-specific, but not in the case of HOPS-specific deletants, indicating that the *slt*$^{-}$ effect on vacuolar size necessitates HOPS.

INTRODUCTION

In the endocytic pathway, traffic between early endosomes (EEs) and late endosomes (LEs)/vacuoles takes place by maturation rather than vesicular traffic (Huotari & Helenius, 2011). The term maturation encompasses the gradual changes in the lipid and protein compositions of endosomes that take place as they are transported by molecular motors. Compositional changes and transport are in turn intertwined with a dynamic balance between fission of membrane subdomains and a progressive increase in size mediated by homotypic fusion. RAB GTPases and their effectors orchestrate the orderly occurrence of all these events (Zerial & McBride, 2001, Behnia & Munro, 2005). Indeed the transition between EEs and LEs involves the progressive acquisition of RAB7 by LEs at the expense of losing RAB5, the master identity determinant of EEs. This process is denoted RAB conversion (Rink *et al.*, 2005, Nordmann *et al.*, 2010, Poteryaev *et al.*, 2010).

Among the multiple effectors of RAB5 and RAB7 are two oligomeric complexes denoted CORVET (class C *core* *v*acuole/*e*ndosome *t*ethering) and HOPS (*ho*motypic fusion and vacuole *p*rotein *s*orting), which have been extensively studied in the yeast *S. cerevisiae* (Peplowska *et al.*, 2007, Brocker *et al.*, 2010, Nordmann *et al.*, 2010, Ostrowicz *et al.*, 2010, Brocker *et al.*, 2012, Balderhaar *et al.*, 2013) and, to a much lesser extent, in the filamentous fungus *A. nidulans* (Abenza *et al.*, 2010, Abenza *et al.*, 2012, López-Berges *et al.*, 2016). CORVET is a *S. cerevisiae* Vps21p and *A. nidulans* RabB^{RAB5} effector (Vps21p and RabB are fungal RAB5 proteins) whereas HOPS is a *S. cerevisiae* Ypt7p and *A. nidulans* RabS^{RAB7} effector. CORVET and HOPS are hetero-hexamers encoded by a subset of *VPS* (required for *v*acuolar *p*rotein *s*orting) genes. The complexes share a common 'core' involving the four 'class C core' components, Vps11, Vps16, Vps18 and Vps33. Therefore, mutations in the corresponding genes affect the two complexes, resulting in strong 'vacuolar protein sorting' defects and severe fragmentation of vacuoles (Raymond *et al.*, 1992, Rieder & Emr, 1997). Besides the 'class C core', the composition of the two complexes is completed with a pair of 'specific' components, Vps8 and Vps3 in CORVET and Vps39 and Vps41 in HOPS (Figure 1A).

The physiological roles of CORVET and HOPS are mediating fusion events at the levels of EEs and LEs/vacuoles, respectively, in two respects: (i) they act as molecular tethers bringing together acceptor and donor membranes (Markgraf *et al.*, 2009, Cabrera *et al.*, 2010, Epp *et al.*, 2011, Balderhaar *et al.*, 2013, Balderhaar & Ungermann, 2013, Kummel & Ungermann, 2014, Kuhlee *et al.*, 2015); (ii) they regulate the actual fusion step through their regulation of the SNARE machinery by the SM (*S*ec1/*M*unc18) 'class C core' component Vps33. SM proteins 'clasp' cognate SNARE bundles to promote their fusogenic action (Sudhof & Rothman, 2009). SM proteins are compartment-specific, such that Vps33 only regulates SNARE bundles of the endovacuolar system (Sato *et al.*, 2000, Seals *et al.*, 2000, Subramanian *et al.*, 2004, López-Berges *et al.*, 2016). Notably Vps33 is unique in that it functions as an integral part of a large multi-protein complex, rather than acting as a separate entity (Balderhaar & Ungermann, 2013). In *S. cerevisiae* Vps33 binds a quaternary SNARE complex containing the Vam3p syntaxin (Lobingier & Merz, 2012). In *A. nidulans*, which like other filamentous fungi does not have a Vam3p homologue, Vps33 regulates PepA^{Pep12}, the only syntaxin acting in EEs and LEs (López-Berges *et al.*, 2016). Actually the recently determined structure of the Vps33/SNARE complex of the filamentous fungus *Chaetomium thermophilum* (Baker *et al.*, 2015) contains a Pep12p rather than a Vam3p orthologue. This structure revealed how Vps33 binds SNAREs to act as a template that facilitates the zippering of the SNARE tetramer.

A notable aspect of the endocytic pathway of *A. nidulans* is that the maturation of EEs to LEs is very important for the physiology of the fungus; consequently ablation of proteins involved in this process is very severely debilitating (Abenza *et al.*, 2010, Calcagno-

Pizarelli *et al.*, 2011, López-Berges *et al.*, 2016). However, growth of mutants deficient in EE maturation can be rescued by inactivation of the *slt* pathway mediating tolerance to cation stress (Calcagno-Pizarelli *et al.*, 2011, Mellado *et al.*, 2015, Mellado *et al.*, 2016). None of our previous studies systematically addressed the roles of CORVET and HOPS, and in particular the predictably crucial roles of 'class C core' components and the possibility that their absence is compensated by inactivation of the *slt* pathway. Here we study the key roles of the specific and 'class C core' CORVET-subunits and confirm that the absence of the latter is also remediable by inactivation of *slt* pathway. However, there is a hierarchy in the extent to which the different mutations can be remediated, with *vps8*Δ and *vps3*Δ being the most, and *vps11*Δ and *vps16*Δ being the least remediable. Notably suppression of *vps8*Δ and *vps3*Δ by *slt*$^{-}$ can only take place if their respective equivalents in HOPS, Vps41 and Vps39, are present, strongly indicating that the i-CORVET and i-HOPS mixed complexes previously identified in yeast can play a physiological role (Peplowska *et al.*, 2007).

MATERIALS AND METHODS

Aspergillus strains, media and molecular genetics

Aspergillus complete (MCA) and synthetic complete (SC) medium containing 1% glucose and 5 mM ammonium tartrate as C and N source, respectively, were routinely used (Cove, 1966). Strain genotypes are listed in Supplementary Table 1. Cassettes for gene deletions were made by fusion PCR, with *A. fumigatus pyrG* (*pyrG*^{Af}) as selective marker (Szewczyk *et al.*, 2006). MAD1739 or MAD3919 (Mellado *et al.*, 2015) (Supplementary Table 1) carrying *pyrG*89 resulting in pyrimidine auxotrophy and *nkuA*Δ preventing non-homologous recombination (Nayak *et al.*, 2005, Szewczyk *et al.*, 2006) were used as recipient strains for transformation. Deletion of *vps3* (AN0763), *vps8* (AN0244), *vps11* (AN12235), *vps16* (AN6911), *vps18* (AN2266), *vps33* (AN2418) and *vps39* (AN10849) genes were carried out as described in the Supplementary Figures 1-7. Gene deletions were verified by diagnostic PCR.

Ultraviolet light (UV)-induced mutagenesis and molecular characterization of *vps33-1*^{ts} suppressors

16 UV-induced *vps33-1*^{ts} suppressors were selected in MAD3262 for growth at 42ºC. In all but two cases, reversion occurred within the *vps33* locus. Meiotic crosses verified that suppressors 7 (*su7vps33-1*^{ts}) and 8 (*su8vps33-1*^{ts}) were extragenic.

Microscopy

For all microscopy experiments, *A. nidulans* cells were grown in 'watch minimal medium' (WMM) (Peñalva, 2005) submerged cultures at 25-28ºC using Lab-Tek chambers (Nalge Nunc International, Rochester, NY) for 16-18 h. To repress *sltA* expression driven by the *thiA* (thiazole synthase, AN3928) promoter, 100 µM (final concentration) of thiamine was added (Calcagno-Pizarelli *et al.*, 2011). Vacuoles were observed with CMAC as described (Abenza *et al.*, 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. MetaMorph software was used for contrast adjustment and z-stack maximal-intensity projections (contrasted, when indicated, with the MetaMorph 'unsharp mask' filter).

RESULTS AND DISCUSSION

Functional interaction between the *slt* pathway and the 'class C core' component Vps33 revealed by random genetic screen

Vps33 (Subramanian *et al.*, 2004) is a key regulator of endovacuolar transport that plays multiple roles acting as a subunit of CORVET and HOPS (Peplowska *et al.*, 2007, Abenza *et al.*, 2010, Ostrowicz *et al.*, 2010, Abenza *et al.*, 2012, Graham *et al.*, 2013). In *A. nidulans* these roles involve the regulation of several SNARE bundles containing the single endovacuolar syntaxin $PepA^{Pep12}$ (López-Berges *et al.*, 2016). Therefore Vps33 was predicted to be crucial for the maturation of EEs into LEs and in turn for the survival of the fungus (Abenza *et al.*, 2010, Calcagno-Pizarelli *et al.*, 2011, Abenza *et al.*, 2012). In agreement, Figure 1B shows that ablation of Vps33, although not strictly lethal, severely impairs growth, leading to very small colonies that are unable to conidiate.

vps33-1^{ts} is a missense mutation leading to severely debilitated growth at 42ºC (Pinar et al., 2013) (Figure 1C and D). To investigate if the essential role of Vps33 can be bypassed by compensatory mutations in other genes we searched for extragenic suppressors rescuing the growth defect displayed by *vps33-1^{ts}* strains at 42ºC. Two such mutations, *su7vps33-1^{ts}* and *su8vps33-1^{ts}* (standard genetic nomenclature for suppressing mutations 7 and 8), were isolated (Figure 2A). *su7vps33-1^{ts}* was mapped, by parasexual analysis (McCully & Forbes, 1965), to chromosome I, with subsequent work revealing tight linkage to *pyroB*100 (AN6141). This region contains *sltB* (AN6132), a gene required for proteolytic conversion of SltA to its functional form for regulating cation homeostasis (Spielvogel *et al.*, 2008, Mellado *et al.*, 2015, Mellado *et al.*, 2016). Notably, *sltB* mutations had been shown to remediate the severe growth defect of ESCRT (*E*ndosomal *S*orting *C*omplex *R*equired for *T*ransport) null mutants blocked in endosomal maturation (Calcagno-Pizarelli *et al.*, 2011). Sequencing of *sltB* in *su7vps33-1^{ts}* identified a missense A1559C mutation leading to Tyr520Ser substitution.

To confirm that this missense mutation in *sltB* is causative of the phenotypic rescue we crossed a strain carrying *sltB*2, a deletion spanning the entire *sltB* region (Mellado *et al.*, 2015) with a *su7vps33-1^{ts} vps33-1^{ts}* double mutant strain and, as control, with a *su8vps33-1^{ts} vps33-1^{ts}* strain. If *su7vps33-1^{ts}* and *sltB*2 were allelic, the progeny of the former cross should not contain any single *vps33-1^{ts}* strain. This progeny included strains showing wt (i.e. *sltB*2 or *su7vps33-1^{ts}* single mutants) or 'suppressed' growth, with two different levels of suppression depending on whether progeny were *su7vps33-1^{ts}* and *vps33-1^{ts}* or *sltB2* and *vps33-1^{ts}* double mutants. However, no progeny displayed unsuppressed *vps33-1^{ts}* growth (Figure 2B), consistent with *su7vps33-1^{ts}* being allelic to *sltB2*. We therefore renamed *su7vps33-1^{ts}* as *sltB100.* In contrast, the progeny of the cross between *sltB*2 and *su8vps33-1^{ts} vps33^{ts}* parental strains contained single unsuppressed *vps33-1^{ts}* strains, which indicates that *sltB2* and *su8vps33-1^{ts}* are not allelic. Notably, in this latter cross we found a new class of 'rescued' *vps33-1^{ts}* colonies, whose conidiation (at 42ºC) was better than that of *vps33-1^{ts} su8vps33-1^{ts}* and *vps33-1^{ts} sltB2* double mutants. This class very likely corresponds to *sltB*2 *su8vps33-1^{ts} vps33-1^{ts}* triple mutant progeny (Figure 2C), which suggests that there are two additive mechanisms of bypassing *vps33-1^{ts}*, involving *su8vps33-1^{ts}* and *sltB*, respectively. Mapping of *su8vps33-1^{ts}* to chromosome VIII established that it is not a *sltA^{-}* allele (*sltA* is located on chromosome VI), suggesting that the mechanism by which the mutation rescues *vps33-1^{ts}* is apparently independent of the *slt* pathway. This mechanism is currently under investigation.

In view of the fact that *su7vps33-1*^{ts} is a *sltB* allele, we asked whether *sltA*Δ (Spielvogel *et al.*, 2008) or *sltB*Δ (Mellado *et al.*, 2015) rescued growth of *vps33-1*^{ts} at 42ºC. Indeed both *sltA*Δ and *sltB*Δ did, albeit to a lesser extent than *sltB100* (*su7vps33-1*^{ts}) (Figure 2D), in agreement with the report that, frequently, *sltA* and *sltB* partial loss-of-function mutations are better suppressors of ESCRT mutations than are the null alleles (Calcagno-Pizarelli et al., 2011). As discussed below *vps33*Δ, like *vps33-1*^{ts}, was rescued by *sltA*Δ, demonstrating that suppression by *slt*$^{-}$ is not allele specific.

sltAΔ partially rescues the severe growth defects resulting from CORVET null mutations

*vps8*Δ removing a CORVET-specific component, and *vps18*Δ removing a CORVET/HOPS 'class C core' subunit were known to cause a severely debilitating phenotype, similar to that of *vps33*Δ (Abenza *et al.*, 2010, Abenza *et al.*, 2012)[note that *vps18* has also been named *digA* because a partial loss-of-function mutation in the gene results in dichotomous growth (Geissenhoner *et al.*, 2001)]. Prompted by these reports we compared the growth phenotypes of *vps8*Δ and *vps18*Δ with those resulting from ablation of the any of the remaining 'class C core' components Vps11, Vps16, and Vps33, and of the other CORVET-specific component Vps3. We also asked whether *slt*$^{-}$ mutations also rescued these growth phenotypes. Therefore we systematically deleted the corresponding genes in wt and *sltA*Δ backgrounds (for details see Supplemental Figures 1-6).

In a *sltA*$^{+}$ background *vps11*Δ, *vps16*Δ, *vps18*Δ, *vps33*Δ, *vps8*Δ and *vps3*Δ alleles phenotypically resembled each other in their severe growth defects (Figure 3A), consistent with the proteins acting in a complex. This result further confirmed that the maturation of EEs, which requires CORVET, is important for *A. nidulans* to thrive normally. Notably, growth of all these mutants was substantially improved by *sltA*Δ, although the mutants fell in two different classes according to the degree of remediation: *vps3*Δ and *vps8*Δ were similarly and quite efficiently rescued, whereas the 'class C core' null alleles were less so (Figure 3B). The physiological interpretation of this finding will be discussed later.

Further differences showed up among the four 'core' alleles upon prolonged storage of culture plates at room temperature: *vps33*Δ and *vps16*Δ were much more efficiently rescued by *sltA*Δ than *vps11*Δ or *vps18*Δ, which were only very weakly rescued (Supplemental Figure 8). The stronger suppression of *vps33*Δ and *vps16*Δ is consistent both with the molecular architecture of HOPS and with the subunit interactions reported for HOPS and CORVET subunits (Ostrowicz *et al.*, 2010, Brocker *et al.*, 2012)(Figure 3C): Vps33 is attached to the complex via Vps16 and, in the case of HOPS (and predictably in CORVET), *vps33*Δ does not disorganize the complex, potentially leaving the other functions intact. Moreover, the absence of Vps33 might be partially fulfilled by the Vps45 SM [note that in *A. nidulans* PepA^{Pep12} is the sole syntaxin operating across the endocytic pathway, and that Vps45, in addition to Vps33, regulates PepA^{Pep12} (López-Berges *et al.*, 2016)].

Exploring the physiological roles of i-CORVET and i-HOPS intermediary complexes

The finding that *vps8*Δ and *vps3*Δ null alleles were the most efficiently rescued by *sltA*Δ led us to hypothesize that the physiological roles of Vps3 and Vps8 might be partially provided by their functional equivalents in HOPS, Vps39 and Vps41, respectively. It is notable that in addition to CORVET and HOPS, two intermediate 'hybrid' complexes are

present in low abundance in *S. cerevisiae* (Peplowska *et al.*, 2007, Ostrowicz *et al.*, 2010) (Figure 4A). i-CORVET, which has been actually isolated from *vps8*Δ mutant yeast cells, consists of all four 'class C core' subunits plus Vps3 and Vps41 (Peplowska *et al.*, 2007). The second complex consists of 'class C core' subunits plus Vps8 and Vps39. It has been denoted i-HOPS (Figure 4A), and even though it could not be isolated, its existence was inferred from co-purification of a proportion of Vps8 with a complex containing Vps39 (Peplowska *et al.*, 2007). Thus our observations might be taken to imply that the hybrid (intermediate) complexes detected by Ungermann and co-workers may indeed have physiological significance (see below). We addressed this possibility genetically.

Contrasting with the severely debilitating effects of ablating CORVET's Vps3 and Vps8, *vps39*Δ and *vps41*Δ mutations removing the 'equivalent' HOPS-specific subunits, Vps39 and Vps41, respectively, have a minor effect on growth (Abenza *et al.*, 2012) (Figure 4B and, for *vps39*Δ, Supplemental Figure 7). Notably, *vps3*Δ showed synthetic negative interactions (i.e. poorer growth of the double mutants than of any of the single mutants) with both *vps39*Δ and *vps41*Δ, which remove HOPS-specific components (Figure 4B). Similarly *vps8*Δ also showed synthetic negative interactions (Figure 4B) with *vps39*Δ and *vps41*Δ. However, when the ability of *sltA*Δ to remediate the severe growth defects of double mutant strains carrying *vps3*Δ or *vps8*Δ was tested, the otherwise phenotypically comparable mutant combinations fell in two classes: the *vps3*Δ *vps41*Δ and *vps8*Δ *vps39*Δ double mutants were remediable by *sltA*Δ, whereas the *vps3*Δ *vps39*Δ and *vps8*Δ *vps41*Δ double mutants were not (Figure 4B). Therefore remediation by *sltA*Δ does not work if the two components (i.e. the one in CORVET and the one in HOPS) of functionally equivalent pairs (i.e. Vps3 and Vps39 or Vps8 and Vps41) are missing simultaneously or, in other words, double mutants involving Vps3 or Vps8 are remediable by *sltA*Δ only of they have capacity to form i-HOPS (*vps3*Δ *vps41*Δ) or i-CORVET (*vps8*Δ *vps39*Δ), which suggests that these i-complexes would be physiologically relevant.

Deletion of *A. nidulans* CORVET-specific components results in a class B phenotype that is remediable by *sltA*Δ

In *S. cerevisiae* deletion of genes mediating the maturation of EEs to LEs including those encoding the RAB5 orthologue Vps21p and the specific subunits Vps3p and Vps8p of its effector CORVET result in a class D phenotype characterized by abnormally large vacuoles (Bowers & Stevens, 2005, Cabrera *et al.*, 2013). In marked contrast, *A. nidulans rabB*Δ ablating the Vps21p orthologue, $RabB^{RAB5}$, leads to small vacuoles characteristic of the yeast 'class B', rather than 'class D', vacuolar phenotype (Abenza *et al.*, 2012). This class B/small vacuole phenotype is shared by *A. nidulans* ESCRT deletions blocking the multivesicular body (MVB) pathway (Calcagno-Pizarelli et al., 2011). Like the associated growth defect, the class B/small vacuole phenotype of ESCRT nulls is remediable by *sltA*$^{-}$ mutations (Calcagno-Pizarelli et al., 2011). However, the severe growth defects resulting from certain *vps*Δ mutations are not merely a consequence of their small vacuole size phenotype because $rabS^{RAB7}$Δ and *vps41*Δ mutants display a class B/small vacuole phenotype yet they do not result in growth defects (Abenza *et al.*, 2012, Pinar *et al.*, 2013) (Figure 4B).

We studied, using CMAC staining, the vacuolar phenotype of the CORVET-specific mutants *vps3*Δ and *vps8*Δ. Both mutants contained numerous small vacuoles and therefore have a class B/small vacuole phenotype, which was indeed remediated by *sltA*Δ (Figure 5A). These data establish that vacuolar size remediation does not require the tethering activity of CORVET (Balderhaar *et al.*, 2013). To buttress this result we generated a *vps8*Δ strain carrying a conditional expression allele of *sltA* based on the thiazole synthase gene promoter ($thiA^{p}$*::sltA*)(Calcagno-Pizarelli et al., 2011). This strain

316 also expressed a GFP-tagged version of the endovacuolar syntaxin PepA^{Pep12} to label
the membrane of the vacuoles (López-Berges *et al.*, 2016). When cultured without
318 thiamine (thus *sltA*$^{+}$ conditions), this strain showed class B vacuoles, like its *vps8*Δ
parental. However, the size and number of vacuoles was restored to normal when
320 thiamine was added, further demonstrating that *sltA*$^{-}$ conditions remediates the class B
phenotype of *vps8*Δ (Figure 5B).
322

The class B vacuolar phenotype of HOPS-specific mutants cannot be remediated
324 **by *sltA*Δ**

326 Deletion of the gene encoding the HOPS-specific components Vps41 (Abenza *et al.*,
2012) and Vps39 (Figure 5C) also gave rise to numerous and small vacuoles.
328 Remarkably, unlike the case of *vps3*Δ or *vps8*Δ CORVET mutants, the class B/small
vacuole phenotype of *vps41*Δ or *vps39*Δ HOPS mutants could not be remediated by
330 *sltA*Δ. Together with the above data, this finding indicates that *sltA*$^{-}$-mediated restoration
of the normal vacuole size in mutants with fragmented vacuoles requires HOPS but not
332 CORVET. The *A. nidulans vps33*Δ affecting both HOPS and CORVET shows a class
B/small vacuole phenotype that, as expected from the above conclusion, could not be
334 remediated by *sltA*Δ (Figure 5D). The fact that the *vps33*Δ growth phenotype is
remediable whereas the vacuolar phenotype is not further confirms that the mechanism
336 of remediation is unrelated to the vacuolar size.

338 **CONCLUDING REMARKS**

340 Here we extend our previous observations to show that null mutations affecting any of
the six *A. nidulans* CORVET components (four 'class C core', shared with HOPS, and
342 two specific) are severely debilitating, and that their phenotypes are remediable to
different extents by downregulation of a fungal-specific salt-tolerance pathway. The
344 CORVET specific mutations *vps3*Δ and *vps8*Δ are the most remediable. This feature has
been already exploited as the means to isolate novel *sltA*$^{-}$ and *sltB*$^{-}$ alleles that arise
346 spontaneously on plates as double mutant sectors with improved growth relative to the
*vps3*Δ parental (Mellado *et al.*, 2015). The fact that *sltA*Δ ameliorates poor growth
348 resulting from inactivating functions as diverse as those of ESCRTs, of PepA^{Pep12}, of its
SM protein Vps45 or of CORVET-specific and 'class C core' components indicates that it
350 is the physiologically crucial endosomal maturation process itself which is bypassed, and
not a particular function of any given protein.
352

Although the mechanism governing this *slt*$^{-}$-dependent remediation remains enigmatic,
354 the key phenomenology is starting to be outlined. Clearly it cannot be directly connected
with the impairment of the canonical roles of the *slt* pathway in tolerance to cation stress.
356 For example, *su8vps33-1*^{ts}, which does not cause '*slt* phenotypes' such as intolerance to
NaCl or alkaline pH rescues *vps33-1*^{ts} to a much greater extent than the *sltB*2 null allele
358 (Figure 2C). Furthermore, the degree at which different *slt* mutations rescue the growth
phenotypes associated with *vps*Δ alleles does not correlate with the hypersensitivity to
360 cation stress that they cause (Calcagno-Pizarelli *et al.*, 2011, Mellado *et al.*, 2015). An
important observation concerns the *slt*$^{-}$-mediated amendment of the abnormally small
362 vacuole size resulting from mutations impairing the endovacuolar pathway. The small
vacuole phenotype of HOPS-specific mutants cannot be remediated by *slt*$^{-}$, suggesting
364 that vacuolar size correction requires HOPS (Figure 5D). A highly speculative possibility
is that one effect of *slt*$^{-}$ mutations might be promoting HOPS-dependent fusion between
366 small vacuoles. It is worth noting that *sltA*Δ itself results in hypertrophy of the vacuolar
system, that two vacuolar calcium pumps are up-regulated in *sltA*Δ strains which have a

368 borderline Ca^{2+} auxotrophy (Findon *et al.*, 2010) and that calcium plays an important role
in homotypic vacuolar fusion (Hay, 2007).
370
It is remarkable that mutations affecting endosomal maturation have diametrically
372 opposite effects on vacuolar size in *A. nidulans* (abnormally small vacuoles) and *S.*
cerevisiae (abnormally large ones). This paradox might be explained by the absence of
374 the yeast vacuolar syntaxin Vam3p in filamentous fungi (López-Berges *et al.*, 2016).
Yeast Vam3p reaches the vacuole directly from the Golgi (Cowles *et al.*, 1997). In
376 contrast, *A. nidulans* $PepA^{Pep12}$ playing the roles of yeast Pep12p and Vam3p reaches
the vacuole via early endosomes, such that impairing the maturation of these
378 endosomes impedes normal traffic of $PepA^{Pep12}$ to later compartments, precluding
homotypic fusion and ultimately leading to small vacuoles.
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