Endosomal maturation by Rab conversion in Aspergillus nidulans is coupled to dynein-mediated basipetal movement

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**ABSTRACT** We exploit the ease with which highly motile early endosomes are distinguished from static late endosomes in order to study Aspergillus nidulans endosomal traffic. Rab\(^{S_{Rab7}}\) mediates homotypic fusion of late endosomes/vacuoles in a homotypic fusion- and vacuole protein sorting/Vps41–dependent manner. Progression across the endocytic pathway involves endosomal maturation because the end products of the pathway in the absence of Rab\(^{S_{Rab7}}\) are minivacuoles that are competent in multivesicular body sorting and cargo degradation but retain early endosomal features, such as the ability to undergo long-distance movement and propensity to accumulate in the tip region if dynein function is impaired. Without Rab\(^{S_{Rab7}}\), early endosomal Rab5—RabA and RabB—reach minivacuoles, in agreement with the view that Rab7 homologues facilitate the release of Rab5 homologues from endosomes. Rab\(^{S_{Rab7}}\) is recruited to membranes already at the stage of late endosomes still lacking vacuolar morphology, but the transition between early and late endosomes is sharp, as only in a minor proportion of examples are RabA/RabB and Rab\(^{S_{Rab7}}\) detectable in the same—frequently the less motile—structures. This early-to-late endosome/vacuole transition is coupled to dynein-dependent movement away from the tip, resembling the periphery-to-center traffic of endosomes accompanying mammalian cell endosomal maturation. Genetic studies establish that endosomal maturation is essential, whereas homotypic vacuolar fusion is not.

**INTRODUCTION**

The mechanisms by which membrane and associated proteins traffic across the endocytic pathway toward degradative organelles (metazoan lysosomes and fungal vacuoles) have been intensively investigated (Huotari and Helenius, 2011). Rather than being vesicle mediated, traffic between early endosomes (EEs) and late endosomes (LEs) occurs by maturation. According to this view, EEs, which receive biosynthetic traffic from the Golgi, progressively undergo changes in luminal pH and composition as they fuse homotypically to give rise to larger organelles. Simultaneously, portions of endosomal membranes that are sorted into the multivesicular body pathway bud inward, thereby delivering lipids and their associated proteins into the lumen of the organelle. Thus maturation results in organelles that are larger than EEs and display a characteristic multivesicular appearance. These multivesicular, “late” endosomes undergo further fusion between themselves and with the vacuoles/lysosomes, thus making their cargo accessible to digestion by the vacuolar/lysosomal hydrolases.

Rabs are small GTPases that, when switched to their GTP conformation, associate with intracellular membranes, recruiting to them “effector” interacting proteins from the cytosol (Behnia and Munro, 2005). These effectors include tethers, soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) regulators, and lipid-modifying enzymes that are major determinants of membrane identity (Zerial and McBride, 2001; Pfeffer and Aivazian, 2004).
Owing to the high specificity of any given Rab for a certain membrane, Rabs have been extensively used in membrane traffic studies, including endosomal maturation itself, as the transition between EEs and LEs appears to be mediated by Rab conversion (Rink et al., 2005; Segev, 2011). In its simplest version, Rab conversion implies that early endosomal membrane domains containing Rab5 are converted into late endosomal domains containing Rab7. As a result, Rab7 effectors substitute for Rab5 effectors, leading to a shift in compartmental identity toward LEs. Replacement of Rab5 by Rab7 on endosomes requires that the positive feedback loops that stabilize Rab5 domains (Zerial and McBride, 2001) need to be inactivated, and the mechanisms facilitating Rab7 recruitment need to be activated coordinately (Rink et al., 2005; Nordmann et al., 2010; Segev, 2011).

Major insight into the mechanisms of endosomal maturation came from work with Saccharomyces cerevisiae (Price et al., 2000; Peplovska et al., 2007; Markgraf et al., 2009; Brocker et al., 2010; Cabrera et al., 2010; Nordmann et al., 2010; Ostrowicz et al., 2010). Endosomal Rab conversion results in substitution of the Vps21p (i.e., Rab5) effector class C core vacuole/endosome tethering (CORVET) complex (Peplovska et al., 2007) by the Vps7p (i.e., Rab7) effector homotypic fusion and vacuole protein sorting (HOPS) complex (Seals et al., 2000). However, the actual mechanism by which HOPS substitutes for CORVET has not been established. Both HOPS and CORVET are multiprotein complexes that share four class C proteins—Vps11p, Vps16p, Vps18p, and Vps33p (Rieder and Emr, 1997)—and only differ in two specific components each—Vps8p and Vps3p for CORVET and Vps41 and Vps39 for HOPS.

A hurdle hindering correlation between subcellular and mechanistic studies of endosomes is implicit in the maturation concept: by definition, the distinction between EEs and LEs is unavoidably blurred (Huotari and Helenius, 2011). The basidiomycete Ustilago maydis and the ascomycete Aspergillus nidulans are ideally suited for studying endosomal maturation because their EEs can be unequivocally identified by their characteristic long-distance bidirectional movement on microtubule-dependent motors (Wedlich-Soldner et al., 2002; Lenz et al., 2006; Steinberg, 2007; Zekert and Fischer, 2008; Abenza et al., 2009, 2010; Hervás-Aguilar et al., 2010; Peña, 2011, 2010; Chung et al., 2011).

A. nidulans has two Rab5 paralogues—RabA and RabB—in addition to Rab7 homologue (Ohsumi et al., 2002; Sánchez-Ferrero and Peña, 2006), which we denoted RabB (Rab seven, RabS). The CORVET complex is an effector of RabB and, to a lesser extent, of RabA (Abenza et al., 2010). Whether HOPS is a RabS effector is unknown. Because maturation involves HOPS-mediated coalescence of EEs into progressively larger endosomes (Markgraf et al., 2009), it seems plausible that the resulting increase in size inevitably results in decreased motility. Such decreased motility would be a bona fide feature of LEs. Indeed, overexpression of the CORVET recruiters RabA/RabB leads to formation of endosomal aggregates with reduced motility and multivesicular appearance (a landmark of LEs; Abenza et al., 2009, 2010; Griffith et al., 2011). However, as discussed by Huotari and Helenius (2011), the presence of internal vesicles cannot be the sole criterion to define LEs. Indeed ESCRT-III appears to operate in A. nidulans EEs (Galindo et al., 2007; Hervás-Aguilar et al., 2010). LEs and vacuoles can be visualized with 7-amino-4-chloromethyl-coumarin (CMAC), a fluorescent compound that labels the lumen of hydrolase-containing acidic organelles, including vacuoles and smaller structures. Here we use the term “vacuole” to refer to spherical CMAC-positive organelles whose limiting membrane is stainable with FM4-64 (Peña, 2005). However, the limiting membrane/shape of the smaller CMAC-positive structures cannot be resolved by optical microscopy. Thus we will use the generic term “LE/vacuole” to collectively refer to all “mature” structures that are CMAC positive, irrespective of their size.

Here we characterize in detail RabS effector, and demonstrate that it localizes to vacuoles and to smaller structures, that HOPS is a RabS effector, and that deletion of rabS leads to the formation of small yet proteolytically competent CMAC-positive structures that, unlike normal vacuoles, undergo long-distance movement and contain early endosomal RabS in their membranes. Time-lapse analyses showed that the transition between RabA/B-containing EEs and RabS structures is sharp, although colocalization can be detected on large (and thus less motile) endosomal aggregates. Dynin impairment results in the accumulation of vacuoles in a region near the tip that is normally devoid of them. Taken together, our data support a model in which endosomal maturation progresses as endosomes move away from the tip, losing motility as they enlarge through homotypic fusion.

RESULTS

RabS localizes to the vacuolar membranes

We studied the localization of green fluorescent protein (GFP)—tagged or mCherry-tagged versions of RabS with constructs containing the rabS promoter or the regulatable promoter alcA. All constructs were single-copy integrated into the genome by targeted recombination (Calcagno-Pizzarelli et al., 2007). Anti-GFP Western analysis, used to determine the levels of GFP-RabS expression obtained with the alcA driver, showed that these were below detection on glucose (alcA-repressing conditions), very high on ethanol (alcA-inducing conditions), and low on 0.1% fructose (noninduced, nonrepressed alcA conditions; Figure 1A). AlcA-driven fructose levels were similar or slightly below those driven by the rabS reporter (Figure 1B). Whether GFP or mCherry tagged, overexpressed (ethanol; Figure 1C) or expressed to approximately physiological levels (fructose; Figure 1D), RabS robustly localizes to the membrane or spherical vacuoles whose lumen is stainable with the vacuolar tracer CMAC.

Rabs associate with membranes in their GTP conformation (Behnia and Munro, 2005). On GTP hydrolysis, GDP-Rabs are extracted to the cytosol by the GDP dissociation inhibitor (GDI; encoded by A. nidulans gdiA). Figure 1F shows that the vacuolar membrane localization of RabS is strictly dependent on the nucleotide switch of the GTPase, as mutant Thr22Asn GFP-RabS, locked in the GDP conformation, is cytosolic (Figure 1F), even though the mutation does not affect the steady-state protein levels (Figure 1E). This shows that GFP-RabS reflects the physiological localization of RabS.

The size and relative abundance of vacuoles increase with the distance to the tip

We exploited the fact that RabS specifically labels vacuoles to measure their distribution along hyphae. We determined the percentage of cell projection surface occupied by vacuoles in 30- to 60-μm-long germings (n = 33), which were segmented into five zones (Figure 2A). Vacuoles occupied, on average, 40% of the conidiospore projection but only 9% of the region nearest to the tip. The regions between the tip and the base showed intermediate scores, the overall tendency being that vacuole-occupied surface increases with the distance to the tip. This increase correlates with a parallel increase in vacuolar diameter (Figure 1C; Peña, 2005; Findon et al., 2010). Thus the biogenesis of vacuoles is connected to the relative position within hyphae, such that tip-proximal regions are...
relatively devoid of these organelles. Vacuoles rarely underwent long-distance movements (Figure 3; see later discussion for a detailed consideration), making unlikely that the mechanism underlying their asymmetrical distribution involves basipetal transport. The strict and robust GFP-RabS\textsuperscript{Rab7} localization to vacuolar membranes facilitated time-lapse studies permitting visualization of vacuoles undergoing fusion (Supplemental Figure S1).

**FIGURE 1:** Subcellular localization of RabS\textsuperscript{Rab7}. (A) Anti-GFP Western blot analysis showing the different steady-state levels of GFP-RabS\textsuperscript{Rab7} achieved under repressing (1% glucose, Glc), noninducing, nonrepressing (0.1% fructose, Frc) and inducing (1% ethanol, EtOH) conditions for the \textit{alcA}\textsuperscript{p} driver. Actin was used as loading control. (B) GFP-RabS\textsuperscript{Rab7} levels driven by the physiological promoter compared with those obtained with \textit{alcA}\textsuperscript{p}. (C) Vacuolar localization of GFP-RabS\textsuperscript{Rab7} \textit{(alcA}\textsuperscript{p} driver, ethanol conditions). (D) Vacuolar localization of GFP-RabS\textsuperscript{Rab7} \textit{(alcA}\textsuperscript{p} driver, fructose conditions). (E) Western blot demonstrating that Thr22Asn substitution does not affect the steady-state level of GFP-RabS\textsuperscript{Rab7} (T22N). (F) GFP-RabS\textsuperscript{Rab7} (T22N) locked in the GDP conformation is cytosolic. Bars, 5 μm.

**FIGURE 2:** \textit{A. nidulans} vacuolar distribution. Pep12 is present in EEs and LEs/vacuoles. (A) Vacuolar distribution: 30- to 60-μm germlings were arbitrarily divided into the “conidium” and four “rectangular” sections of approximately equivalent length, as indicated. The percentage of the total projection area occupied by vacuoles in each section was plotted in \( n = 33 \) germlings. (B) mCherry-RabS\textsuperscript{Rab7} colocalizes with GFP-Pep12 on vacuoles. However, GFP-Pep12 additionally localizes to punctate structures that are not labeled by mCherry-RabS\textsuperscript{Rab7}. Regions of interest are shown at double magnification in the indicated red, green, and merge channels. (C) Kymograph of a 4-frame/s time-lapse sequence of a cell coexpressing GFP-Pep12 and the EE marker mCherry-RabA. Pep12 and RabA colocalize in the population of rapidly moving EEs and in less abundant larger and static RabA-containing structures but not on vacuoles, which do not contain RabA.

**Partial overlap of the target-SNARE Pep12 with RabS\textsuperscript{Rab7}**

The \textit{A. nidulans} orthologue of \textit{S. cerevisiae} Pep12p is the only syntaxin across the endovacuolar system (Sánchez-Ferrero and Peñalva,
rabSΔ results in proteolytically competent minivacuoles capable of undergoing movement

rabS disruption results in small vacuoles (Ohsumi et al., 2002). Strains carrying rabSΔ indeed showed minute vacuoles (CMAC staining; Figure 3B) whose small lumen is hardly resolvable by optical microscopy (using GFP-Pep12 to label their membranes; Figure 3C). Thus, in agreement with previous data and with work in S. cerevisiae Ypt7p, RabSΔ is involved in the biogenesis of “normal-sized” vacuoles. rabSΔ “minivacuoles” show two noteworthy features: 1) They are evenly distributed across hyphae, unlike wild-type vacuoles (Figure 3, D and E), and 2) a proportion of mutant minivacuoles undergoes long-range movements similar to those of EEs (reflected by diagonal lines in kymographs), contrasting with the essentially immobile wild-type vacuoles.

RabSΔ strictly cosegregates with the growth/condiation defects in the progeny of crosses established that these phenotypic features are solely attributable to the rabSΔ allele.

FIGURE 3: rabSΔ results in abnormally motile minivacuoles. (A) Growth phenotypes of the indicated strains at the indicated media and temperatures. MCA, complete medium. SC, synthetic complete medium. (B) CMAC and (C) GFP-Pep12 visualization of rabSΔ minivacuoles. All images are depicted at the same magnification. Bar, 2 μm. (D) Still frames (blue) and kymographs (inverted contrast, shown at the same scale as still images) correspond to 10-s time-lapse series of CMAC-stained vacuoles. Note the multiple diagonal lines seen in the rabSΔ example. Both images are at the same magnification. Bar, 5 μm.

2006; Findon et al., 2010; S. cerevisiae has two—Pep12p for pre-vacuolar endosomes and Vam3p for vacuoles). mCherry-RabSΔ strictly colocalizes with GFP-Pep12, but the reverse is not true, as GFP-Pep12 labels punctate structures that do not contain RabSΔ (Figure 2B). These punctate structures undergo long-distance bidirectional movements, suggesting that they are EEs. Thus we constructed a strain coexpressing GFP-Pep12 and the EE marker mCherry-RabA and recorded time-lapse sequences (~4 frames/s) simultaneously in the GFP and mCherry channels. These sequences were analyzed with kymographs in which the y-axis represents the time scale. Thus static and short-range moving structures appear as vertical lines, whereas fast-moving structures appear as diagonal lines whose slope reflects the speed/direction and length reflects the range of movement of any given particle. Figure 2C shows that GFP-Pep12 localizes to static vacuoles (bright vertical lines) that do not contain RabA, to static structures containing RabA (LEs; see later discussion), and, in addition, to a population of small, bidirectionally motile structures (thus EEs; Figure 2C diagonal lines; Supplemental Movie S1) in which it colocalizes with RabA. This important finding indicates that Pep12 plays roles at the levels of the A. nidulans EEs, LEs, and vacuoles.

rabSΔ slightly impairs vegetative growth and results in a temperature-dependent defect in conidiation

We constructed a null rabSΔ allele, which results in a minor vegetative growth defect at any of the three temperatures tested (30°, 37°, and 42°C), more noticeable on synthetic than on rich medium (Figure 3A). Its most conspicuous phenotype was the nearly complete absence of conidiation at 42°C (Figure 3A). This indicates that Pep12 strictly cosegregates with the growth/condiation defects in the progeny of crosses established that these phenotypic features are solely attributable to the rabSΔ allele.

We next determined whether rabSΔ minivacuoles are competent in proteolysis of an endocytic cargo, the plasma membrane amino acid permease AgtA. AgtA is expressed on glutamate medium, localized to the plasma membrane and vacuoles (Figure 4A). If glutamate-cultured cells are shifted to ammonium, AgtA synthesis is strongly repressed, and the plasma membrane-resident pool of the transporter is delivered to the vacuole via endocytosis (Apostolaki et al., 2009). In rabSΔ cells, AgtA-GFP is also delivered to the lumen of (mini-) vacuoles (Figure 4A). Western blots revealed similar kinetics of AgtA-GFP degradation in rabSΔ and rabBΔ cells (Figure 4B). Thus rabSΔ does not affect the sorting of cargoes into the MVB pathway, and minivacuoles are proteolytically competent.

RabSΔ recruits HOPS

We next used glutathione S-transferase (GST)–RabSΔ, loaded with GDP or GTP-γ-S, and cell extracts containing endogenously 3x-hemagglutinin (HA)-tagged interactors in pull-down experiments (Figure 5A). GdiA was specifically and efficiently pulled down by GDP-loaded RabSΔ, indicating that GTP-γ-S shifts RabSΔ toward a conformation that prevents its interaction with the Rab-GDP effector GdiA (Figure 5A). The fusogenic activity of Ypt7p involves HOPS. Vps41p is the Ypt7p effector subunit in HOPS linking the complex to the GTP-loaded Rab (Cabrera et al., 2010; Ostrowicz et al., 2010). In agreement, A. nidulans Vps41 is specifically pulled down by GTP-γ-S RabSΔ but not at all by RabB, which instead pulled down Vps8 efficiently (Vps8 is the CORVET equivalent of Vps41; Pempolska et al., 2007; Markgraf et al., 2009). In contrast, Vps39, which also showed strict RabSΔ specificity, was preferentially retained by GTP-γ-S RabSΔ but interacted substantially with GDP-RabSΔ (Figure 5A). Given that we used unfractionated “prey extracts,” this finding is consistent with the finding that whereas the Vps39-containing HOPS
These data indicate that γpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγpXγ
Rab7

1894

Subcellular distribution of Rab5s and RabS

To study endosomal Rab transitions, we constructed strains expressing mCherry-RabS

GFP-RabA or GFP-RabB from single-copy alcA-driven transgenes. Using this regulatable (by the carbon source) promoter allowed us exploit three different levels of expression: low (fructose), intermediate (glycerol), and high (ethanol). None of these carbon source conditions led to any detectable effect on colony growth (Supplemental Figure S4), strongly indicating that expression of the fusion proteins does not interfere with the physiology of the endosomal system.

We acquired high-speed time-lapse sequences (10–20 frames/s) of these strains in the red and green channels simultaneously using a Dual-Viewer. Rab5Δ and RabA/RabB showed little colocalization (Figure 7A and Supplemental Movies S4 and S5). Rab5Δ localized to the vacuolar membranes, to relatively static structures, and, occasionally, to moving structures (see later discussion). By contrast, RabA or RabB did not label the vacuolar membranes at all, localizing to moving EEs and to less abundant static structures. As in U. maydis (Schuster et al., 2011), moving RabA/RabB EEs usually traveled for several seconds in one direction before reversing movement in the opposite direction, with (U-shaped tracing in the kymograph; Figure 7B) or without (V-shaped tracings; Figure 8) arresting for some time in between. This behavior reflects the tug-of-war action of dynein and kinesins on the same endosome (Schuster et al., 2011). Time-lapse sequences revealed very unusual examples of vacuolar movement, like the ~6-μm journey of the small vacuole in Figure 7C to dock at a large vacuole (Supplemental Movie S6). This movement was short ranged and slower than that of EEs (compare slopes of green- and red-channel kymographs).

The transition between RabA/RabB and Rab5Δ is sharp

RabB is the major determinant of endosomal maturation (Abenza et al., 2010). Fructose kymographs (Figure 8, A1 and A2) revealed occasional yet rare examples of moving GFP-RabB EEs faintly stained with mCherry-Rab5Δ (Figure 8A2, arrows). In contrast, static structures containing RabB and Rab5Δ were clearly more abundant (Figure 8A1, arrows). Similar overall absence of colocalization in moving structures was seen on glycerol, despite the increased

GFP-Rab5Δ & CMAC in vps41Δ

GFP-Rab5Δ

5 μm

GFP-Rab5Δ

5 μm

FIGURE 6: Genetic studies of endosomal maturation. (A) Growth phenotypes of vps18Δ (encoding a class C subunit shared by the CORVET and HOPS complexes) and vac1Δ (Vac1, also denoted Vps19, is a specific RabB effector), vps18Δ and vac1Δ are very severely debilitating. Note that vps18 was previously called digA. A digA1 early-truncating mutation removing the Vps18 C-terminal RING finger leads to a temperature-dependent growth defect (Geissenhoner et al., 2001). (B) Growth phenotypes at the indicated temperatures of vps41Δ compared with rab5Δ. The two mutations are phenotypically indistinguishable. (C) Both rab5A and rab5Δ result in a synthetic growth defect in double mutants with rab5Δ. rab5Δ also results in a similar synthetic growth defect in double mutants with vps41Δ (rab5A was not tested in combination with vps41Δ). (D) Like rab5Δ, vps41Δ results in minivacuoles. In the absence of Vps41, the limiting membranes of CMAC-containing minivacuoles are labeled with GFP-Rab5Δ. Images of a z-series are shown, with numbers indicating relative z-position in micrometers. For comparison, GFP-Rab5Δ wild-type vacuoles are shown on the right.

GFP-Rab5Δ in wt

5 μm

GFP-Rab5Δ in wt

5 μm
Rab7

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It also localized to aggregates that, albeit with RabB, we only very occasionally detected EEs labeled with RabS\textsubscript{Δ}\textsubscript{Rab7}. Therefore, although these strongly overexpressing conditions are not physiological, these experiments establish that RabS\textsubscript{Δ}\textsubscript{Rab7} can be recruited to endosomal membranes containing high levels of RabB. In contrast, RabB is excluded from vacuolar membranes, where RabS\textsubscript{Δ}\textsubscript{Rab7} is abundant. These data are consistent with a maturation model in which endosomes acquire RabS\textsubscript{Δ}\textsubscript{Rab7} in their late steps of maturation, losing RabB as they become vacuoles.

Similar experiments using GFP-RabA revealed a previously unnoticed feature of RabA endosomes: even under fructose conditions leading to GFP-RabA levels similar to those attained with the endogenous promoter (Abenza et al., 2009), we detected a background of very abundant small, moving endosomes that we did not see with GFP-RabB; their kymograph tracings formed a net of diagonal lines against which vertical lines of more static aggregates were prominent (Figure 8, C and D, Supplemental Figure S5, and Supplemental Movie S9). As with RabB, we only very occasionally detected EEs labeled with RabS\textsubscript{Δ}\textsubscript{Rab7} and RabA. The tracings arrowed in Figure 8D and Supplemental Figure S5B are the only examples among the numerous moving RabA EEs in which RabS\textsubscript{Δ}\textsubscript{Rab7} was detectable. We thus conclude that the transition between RabA/RabB and RabS\textsubscript{Δ}\textsubscript{Rab7} domains is relatively sharp.

**RabB and RabA reach the vacuoles in rabS\textsubscript{Δ}**

rabS\textsubscript{Δ} markedly increases the abundance of RabA/RabB structures. In 30-μm-long projections of wild-type tip regions (n = 8 hyphae), GFP-RabB structures accounted for 4.4 ± 0.6% of the area. This figure was markedly higher (sixfold; 23.3 ± 2.5%) in the mutant. This hypertrophy of the rabS\textsubscript{Δ} endosomal compartment might reflect the inability of LEs to undergo HOPS-mediated fusion or the ability of EE RabB to invade membranes otherwise restricted to RabS\textsubscript{Δ}\textsubscript{Rab7} (or both). One observation lent credence to the “Rab5 invasion” possibility. In the wild type, GFP-RabA/RabB eventually label aggregates of endosomes that are not vacuoles (they are multivesicular, irregularly shaped, and CMAC negative; Abenza et al., 2009, 2010; Griffith et al., 2011). In contrast, in rabS\textsubscript{Δ} cells, both RabA (Supplemental Movie S10) and RabB (Figure 9A) clearly label the membranes of minivacuoles. To further demonstrate this, cells expressing GFP-RabB were pretreated with benomyl (to abolish the microtubule [MT]-dependent motility of minivacuoles) and strongly labeled with RabS\textsubscript{Δ}\textsubscript{Rab7} and RabB-αRab5, and RabB-αRab5, located between two vacuoles. One of the endosomes moves in one direction before shifting toward the opposite direction, but its speed (1.8 μm/s) is lower than the average speed of EEs. Frames correspond to supplemental Movie S7.

**FIGURE 7:** Coimaging of early endosomal Rabs and RabS\textsubscript{Δ}\textsubscript{Rab7}. (A) Still frames showing that RabS\textsubscript{Δ}\textsubscript{Rab7} and RabB or RabA show little colocalization. (B) Examples of static and moving endosomes: RabB (green) and RabS\textsubscript{Δ}\textsubscript{Rab7} (mCherry). A kymograph was plotted across the indicated line. Three static RabB endosomes, one moving EE, and two small vacuoles strongly labeled with RabS\textsubscript{Δ}\textsubscript{Rab7} are shown. The tip-distal static RabB endosome is docked at the most anterior RabS\textsubscript{Δ}\textsubscript{Rab7} vacuole (yellow in the channel merge). The movement of a fourth RabB endosome is schematically represented. (C) One example (arrows) of a basipetally moving small RabS\textsubscript{Δ}\textsubscript{Rab7} vacuole (smV) that docks against a large vacuole (LgV). Kymographs on the right depict how the speed (diagonal slope, 1.34 μm/s) of this moving vacuole is markedly lower than that of RabA endosomes. See Supplemental Movie S6. (D) RabB and RabS\textsubscript{Δ}\textsubscript{Rab7} can coexist on LEs: two LEs containing RabB and RabS\textsubscript{Δ}\textsubscript{Rab7}, located between two vacuoles. One of the endosomes moves in one direction before shifting toward the opposite direction, but its speed (1.8 μm/s) is lower than the average speed of EEs. Frames correspond to supplemental Movie S7.

fluorescence signal. Again, colocalization predominated in the more static structures or in those motile structures that moved for shorter distances and at slower speeds than EEs (Supplemental Figure S5A). Figure 7D and Supplemental Movies S7 and S8 show structures containing RabB and RabS\textsubscript{Δ}\textsubscript{Rab7} moving at 1–1.8 μm/s, more slowly than EEs (–2.5 μm/s; Abenza et al., 2009). We speculate that these double-labeled structures, whose size would impede/retard movement, represent late or mature endosomes where Rab conversion is taking place.

To confirm that RabB and RabS\textsubscript{Δ}\textsubscript{Rab7} can actually coincide on the less motile endosomes, we induced the transgenes with ethanol, which results in strong overexpression of the Rabs. RabB still localized to fast-moving EEs. It also localized to aggregates that, albeit motile, generally moved for shorter distances (Figure 8, B1 and B2). RabS\textsubscript{Δ}\textsubscript{Rab7} was clearly present in a proportion of RabB-containing structures, generally corresponding to these large aggregates (Figure 8, B1 and B2, double arrowed; one “control” RabS\textsubscript{Δ}\textsubscript{Rab7}, negative EE is single arrowed). However, RabB was absent from vacuolar membranes, even though these were strongly positive for RabS\textsubscript{Δ}\textsubscript{Rab7}. These data are consistent with a maturation model in which endosomes acquire RabS\textsubscript{Δ}\textsubscript{Rab7} in their late steps of maturation, losing RabB as they become vacuoles.
and loaded with CMAC. Whereas hardly any overlap of RabB with CMAC was detectable in the wild type, most RabB-containing structures in the rabΔ mutant were CMAC positive (Figure 9B). Because vacuolar membrane labeling by RabA/RabB is never seen in the wild type, these data suggest that displacement of RabB/RabB from EEs once they reach the LE stage requires RabSΔ.

We next tested whether GFP-RabSΔ localization to vacuoles necessitates RabA. GFP-RabSΔ is recruited to the membrane of rabΔ vacuoles, although clearly less efficiently than in the wild type (Figure 9C). This finding is consistent with the interpretation that the vacuolar RabSΔ localization involves CORVET, as RabA is also able to recruit CORVET to endosomes, albeit less efficiently than RabB (note that lethality-causing rabΔrabΔ double deletion cannot be tested).

The role of dynein-mediated basipetal movement in endosomal maturation

Given that the amount and size of vacuoles increase with the distance to the tip, we hypothesized that maturation of endosomes into vacuoles might be coupled to the dynein-mediated movement of endosomes away from the tip (MTs are oriented with their plus ends toward the apex). Thus, if dynein function is compromised, EEs would be expected to mature in the proximity of the tips. Indeed the dynein (heavy chain) nudAΔ mutation caused a pronounced effect on the apicalbasal distribution of GFP-RabSΔ-labeled vacuoles. In nudAΔ germlings (Figure 10A) and hyphae (Figure 10B) there was a marked accumulation of vacuoles near the tip that was never seen in the wild type (wild-type tip-proximal regions are actually devoid of vacuoles). Clusters of nudAΔ vacuoles did not invade the actual tip of hyphae (Figure 10B). These findings strongly support a model according to which endosomes mature to LEs that become engaged in homotypic fusion events to coalesce into larger vacuoles as they move away from the tip (Figure 11). We also constructed nudAΔrabΔ double-mutant strains expressing GFP-RabSΔ. This double mutation does not prevent the vacuolar localization of RabSΔ (as noted, RabA is able to recruit CORVET), even though these strains display the characteristic nudAΔ accumulation of vacuoles near the tip (Supplemental Figure S6B).

We conducted additional experiments using CMAC to visualize vacuoles in cells that do not express any fluorescent Rab. nudAΔ hyphal tip cells indeed accumulate clusters of small vacuoles within the apicalmost 10- to 20-μm region. These clusters were absent in the wild type (Figure 10, D and E), in agreement with data presented earlier. We also noticed that in rabSΔ cells, the otherwise ubiquitous minivacuoles are absent from the tip (Figure 10, D and E), suggesting that they are excluded from this region, predictably by dynein-mediated transport. If this were true, in nudAΔ cells minivacuoles should invade the tip, forming a minivacuolar analogue of the EE cluster characteristic of dynein-deficient hyphal tips (Lenz et al.,

mCherry-RabSΔ are indicated with red and green arrows. (B2) One RabB endosome that does not contain mCherry-RabSΔ is indicated with a single green arrow. GFP-RabA: (C) Virtually no colocalization of GFP-RabA and mCherry-RabSΔ is seen in the numerous examples of moving endosomes detected across this 60-μm-long region. (D) The endosome on the left (red and green arrows of equal size), containing GFP-RabA and mCherry-RabSΔ, moves toward the tip until it docks at a LE/vacuole. Two examples of GFP-RabA moving endosomes faintly stained with mCherry-RabSΔ are indicated with green (large) and red (small) arrows. Note that moving RabA structures (diagonals in C and D) are clearly more numerous than moving RabB structures (A and B).
FIGURE 9: In rabSΔ strains, RabB invades vacuolar membrane domains normally occupied by RabS
rabSΔ. (A) GFP-RabB in rabSΔ. Inverted contrast images correspond (at double magnification) to the indicated regions of interest in the Nomarski panel. They show how the early endosomal RabB reaches the membrane of rabSΔ minivacuoles. Similar data obtained for GFP-RabA are displayed in Supplemental Movie S10. (B) Microtubules were depolymerized with benomyl. The relative difference of fluorescence is illustrated by the line scans on the right. The inset indicating that CMAC-stained Rab7 vacuolar structures was less apical or, at most, overlapped with the rest of the cell largely devoid of them. Invasion of nudA1Δ tip regions by minivacuoles is very conspicuous in young branches (Supplemental Figure S6A).

Finally, we hypothesized that by impairing EE movement, nudA1 would confine maturation of EEs into LEs/vacuoles within the tip. Thus we examined nudA1 cells coexpressing fluorescent RabB and RabSrabSΔ that had been shifted to the restrictive temperature for 4 h. These cells contained the characteristic nudA1 aggregate of RabB-containing endosomes unable to depart from the tip (Figure 10C) and the also characteristic tip-proximal clusters of RabSrabSΔ vacuolar structures. RabB aggregates and RabSrabSΔ structures were very closely associated and showed partial colocalization (Figure 10C and Supplemental Figure S7). However, in every example analyzed (n = 47 tips examined with Dual-Viewer z-stacks), the cluster of LE/vacuolar structures was less apical or, at most, overlapped with the EE aggregate (Supplemental Figure S7), strongly supporting the view that conversion of RabB EEs into RabSrabSΔ LEs/vacuoles is coupled to basipetal movement of the former.

DISCUSSION

In U. maydis and A. nidulans kinesin-3 and dynein mediate the long-distance movement of EEs (Wedlich-Soldner et al., 2002; Lenz et al., 2006; Zekert and Fischer, 2008; Abenza et al., 2009, 2010). Endocytosis predominates in the tip region (Araujo-Bazán et al., 2008; Taheri-Talesh et al., 2008), which is the major source of EEs (Figure 11). These endosomes move away from the tip using dynein. Thus, in A. nidulans strains deficient in the NudA dynein, in the dynein activator NudF, in the KinA kinesin-1 transporting dynein to MT plus ends, or in the dynactin p25 subunit linking EEs to dynein, EEs aggregate in the tips (Zekert and Fischer, 2008; Abenza et al., 2009, 2010; Zhang et al., 2010, 2011).

In time-course experiments, the endocytic tracer FM4-64 arrives first to motile EEs and, in a posterior stage, to a population of larger, static LEs (Peñalva, 2005; Hervás-Aguilar et al., 2010). Thus the transition from EE to LEs takes place with simultaneous loss of motility. In our model, maturation of EEs is coupled to their movement away from the tip (Figure 11), resembling the situation in mammalian cells, in which Rab5 EEs that form in the cell periphery (the hyphal tip equivalent) move centripetally (i.e., basipetally in fungal hyphae) on dynein while they augment their size (Rink et al., 2005). However, in mammalian cells, LEs/lysosomes also undergo dynein-mediated centripetal transport toward the minus ends of MTs. Recruitment of dynein-dynactin to LEs/lysosomes is mediated by the Rab7 effector RILP (Jordens et al., 2001). RILP homologues are absent from fungi. Thus inefficient dynein recruitment might contribute to the reduced motility of A. nidulans LEs enriched in RabSrabSΔ.

Our model in Figure 11 accounts for the observations that tip-proximal regions contain very few vacuoles (the end product of endosomal maturation) and that vacuolar size increases with the distance to the tip. It is strongly supported by three findings: 1) Vacuoles accumulate near the tip region of nudA1Δ cells; 2) unlike wild-type vacuoles, rabSΔ minivacuoles, which are abnormally motile, are uniformly dispersed across the cytosol; and 3) In nudA1Δ cells, overlap of Rab5 and RabSrabSΔ membranes is detectable in the tip, suggesting that if retrograde EE movement is prevented, endosomes mature on the spot.

The absence of RabSrabSΔ prevents the formation of normalized vacuoles. The resulting minivacuoles have features of EEs: 1) They are able to eventually undergo long-distance movement; 2) they invade the apicalmost regions of nudA1Δ tips, resembling EEs; and 3) they contain EE RabS on their membranes. The finding that RabS invade LE/vacuolar membranes only if RabSrabSΔ is absent is consistent with the models in which maturation of EEs into LEs/vacuoles occurs by Rab conversion (Rink et al., 2005; Peploiwka et al., 2007; Brocker et al., 2010). In a simplified model, this implies that RabS/CORVET membrane domains are converted into Rab7/HOPS domains (Rink et al., 2005). A key regulator of this conversion is a complex of two proteins formed by Mon1p/Ccz1p in S. cerevisiae (Nordmann et al., 2010) and SAND-1/CCZ-1 in C. elegans (Kinchen and Ravichandran, 2010). The findings that SAND-1/CCZ-1 is itself a Rab5 effector (Kinchen and Ravichandran, 2010), that the localization to endosomes of Mon1p/Ccz1p is Vps21p (Rab5) and CORVET dependent (Nordmann et al., 2010), that the complex interacts with HOPS components (Nordmann et al., 2010; Poteryaev et al., 2010), and, of importance, that Mon1p/Ccz1p is the guanine nucleotide exchange factor (GEF) of Vps21p/Ccz1p is the guanine nucleotide exchange factor (GEF) of Mon1p/Ccz1p is the guanine nucleotide exchange factor (GEF) of Mon1p/Ccz1p is the guanine nucleotide exchange factor (GEF) of Mon1p/Ccz1p is the guanine nucleotide exchange factor (GEF) of Mon1p/Ccz1p is the guanine nucleotide exchange factor (GEF) of Mon1p/Ccz1p is the guanine nucleotide exchange factor (GEF) of
LEs whose mobility is constrained by their size. Thus RabS\textsuperscript{rab7} is recruited to membranes already at the level of LEs and it is not confined to vacuoles, in agreement with data in S. cerevisiae (Balderhaar et al., 2010). In contrast, RabA or RabB, both of which reside in EEs, can reach LEs but are normally absent from vacuoles.

Fusion of A. nidulans LEs with vacuoles and among vacuoles themselves is dispensable, as demonstrated by the relatively mild growth defects resulting from rab\textsuperscript{Δ}, vps41\textsuperscript{Δ} (this work) and vps39\textsuperscript{Δ} (Oka et al., 2004).

Our data strongly indicate that the transition between Rab5s and Rab7 is sharp, as examples of rapidly moving RabA/RabB EEs reach vacuoles through endosomes, being able to reach LEs whose mobility is constrained by their size. Thus RabS\textsuperscript{rab7} is recruited to membranes already at the level of LEs and it is not confined to vacuoles, in agreement with data in S. cerevisiae (Balderhaar et al., 2010). In contrast, RabA or RabB, both of which reside in EEs, can reach LEs but are normally absent from vacuoles.

Fusion of A. nidulans LEs with vacuoles and among vacuoles themselves is dispensable, as demonstrated by the relatively mild growth defects resulting from rab\textsuperscript{Δ}, vps41\textsuperscript{Δ} (this work) and vps39\textsuperscript{Δ} (Oka et al., 2004). In contrast, double rab\textsuperscript{AΔ} rab\textsuperscript{BΔ} deletion is lethal (Abenza et al., 2010), and single deletion of each tested gene involved in maturation of EEs into LEs is very severely debilitating. Tested mutations include vps45\textsuperscript{Δ}, vps8\textsuperscript{A} (CORVET; Abenza et al., 2010), vps18\textsuperscript{A} and vac1\textsuperscript{A} (this work), ESCRT\textDelta alleles (single vps27\textsuperscript{Δ}, vps23\textsuperscript{Δ}, vps20\textsuperscript{Δ}, vps24\textsuperscript{Δ}, and vps32\textsuperscript{Δ} mutations), and a conditional expression allele of Vps4 (Rodríguez-Galán et al., 2009; Calcagno-Pizarrelli et al., 2011; Galindo et al., 2012). Of note, the severely debilitating effect of ESCRT\textDelta alleles is suppressible by loss-of-function mutations in the filamentous fungal–specific sltA gene regulating cation homeostasis (Findon et al., 2010; Calcagno-Pizarrelli et al., 2011), suggesting that the essential role of endosomal maturation might be related to cation homeostasis.

rab\textsuperscript{AΔ} minivacuoles are proteolytically competent and contain Pep12. Thus resident proteases or Pep12 must be able to reach vacuoles through endosomes, because the direct AP-3 pathway to the vacuole requires Ypt7p/HOPS/vps41p (Angers and Merz, 2009; Cabrera et al., 2010). In the absence of RabS\textsuperscript{rab}, the formation of minivacuoles possibly occurs through the CORVET-mediated homotypic fusion of endosomes (Markgraf et al., 2009), but CORVET alone appears to be insufficient to sustain further enlargement of minivacuoles. Thus an attractive and as yet untested possibility is that CORVET and HOPS complexes mediate homotypic fusion events but that these events involve partners with different degrees of membrane curvature, depending on the Rab/tether combination.

**MATERIALS AND METHODS**

**Aspergillus media and molecular biology**

Synthetic complete medium (SC; Cove, 1966) contained 1% glucose and 5 mM ammonium tartrate unless otherwise indicated. Complete medium for Aspergillus (MCA) was used for strain maintenance. Strains are listed in Supplemental Table S2 (vps18\textsuperscript{A}, Vac1\textsuperscript{A}, and the double mutants rab\textsuperscript{AΔ}-rab\textsuperscript{SΔ}, rab\textsuperscript{BΔ}-rab\textsuperscript{SΔ}, and vps41\textsuperscript{Δ}-rab\textsuperscript{AΔ} strains were impossible to keep beyond the described experiments due to their severely debilitating phenotype). For growth tests involving different carbon sources, we cultivated the strains on SC containing 5 mM ammonium tartrate and 3% glucose,
Western blotting

To determine levels of GFP-RabS\textsuperscript{Rab7} by Western blotting, we extracted proteins as described (Hervás-Aguilar et al., 2007). GFP fusion proteins were detected using mouse anti-α-GFP (monoclonal cocktail, 1:5000; Roche, Indianapolis, IN). Actin (detected with mouse anti-α-actin from ICN Biomedicals (Irvine, CA) using 1:80,000 dilution of the antibody) was used as loading control. For the extraction of AgtA-GFP we used a reported procedure (Hervás-Aguilar and Peñalva, 2010) involving solubilization of proteins from lyophilized mycelial biomass with NaOH, followed by their precipitation with trichloroacetic acid.

Microscopy

Cells were grown in submerged cultures at 25°C in watch minimal medium (WMM), using Lab-Tek chambers (Nalge Nunci International, Rochester, NY) for 14–16 h before proceeding to microscopy (Pantazopoulou and Peñalva, 2009). To modulate the expression levels of the fluorescent chimeras expressed under the control of alc\textsuperscript{Ac}, we used different carbon sources (Abenza et al., 2009): low levels were attained with 0.1% (wt/vol) fructose, and high levels were obtained using 1% ethanol (vol/vol) or by preculturing cells on 0.02–0.05% (wt/vol) glucose and shifting them to 1% (vol/vol) ethanol for 3–4 h. In RabA-RabS\textsuperscript{Rab7} and in RabB-RabS\textsuperscript{Rab7} colocalization experiments, intermediate expression levels were achieved by culturing cells in WMM containing 1% (vol/vol) glycerol as carbon source. Mature endosomes/vacuoles were detected with CMAC as described (Abenza et al., 2009; Pantazopoulou and Peñalva, 2009). To determine the effects of the nud\textsuperscript{A1} mutation, cells were cultivated at 37°C, which is a semirestrictive temperature, or overnight at 25°C (permissive temperature) and then shifted to 37°C for a few hours before image acquisition. To impede movement of rab\textsuperscript{A} minivaquelos, benomyl was added to WMM at a final concentration of 4.8 µg/ml as described (Abenza et al., 2009). For experiments involving delivery of AgtA-GFP to the vacuole, cells were cultured at 25°C for 14–16 h in WMM containing 5 mM l-glutamate as nitrogen source and then shifted to the same medium in which 5 mM ammonium tartrate substituted for l-glutamate. AgtA-GFP images were taken at the 0 min time point and 80 min after the shift (Abenza et al., 2009, 2010).

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. The microscope was equipped with HCX 63×, 1.4 numerical aperture (NA), and 100×, 1.4 NA, objectives and BrightLine GFP-3035B (Semrock, Rochester, NY), TXRED-4040B (mCherry), and standard filters (Semrock, Rochester, NY), TXRED-4040B (mCherry), and standard filters.

0.1% fructose, or a combination of 1% ethanol and 0.02% glucose during 72 h at the indicated temperatures.

\textit{A. nidulans} vps\textsuperscript{18} (dig\textit{A}), Vac1, vps41, and gdi\textit{A} were identified as AN2266, AN3144, AN4876, and AN5895, respectively, in the \textit{A. nidulans} genomic database. Deletion cassettes were constructed by PCR (Szewczyk et al., 2006), using primers listed in Supplemental Table S3 and \textit{Aspergillus fumigatus} pyr\textsuperscript{G} (for vps18\textit{A}, rab5\textit{A}, vac1\textit{A}, and vps41\textit{A}) or pyr\textsuperscript{Ac} (for a second rab5\textit{A} deletion allele) as selection markers. Recipient strains carried nku\textit{A}, preventing non-homologous recombination (Nayak et al., 2006). Constructs for integrative transformation were confirmed by sequencing. They were targeted to the pyro\textit{A} or arg\textit{B} locus after transformation, using published methodology (Calcagno-Pizarelli et al., 2007; Pantazopoulou and Peñalva, 2009). Single-copy integration was verified by Southern blots.
Affinity purification of RabS$^{Rab7}$ effectors
Affinity purification of effectors using GDP- or GTP-$\gamma$S-GST-Rab$^{Rab7}$ glutathione-Sepharose beads was made as described for GST-RabB. Glutathione-Sepharose 4B beads containing GST-RabS$^{Rab7}$ were incubated with A. nidulans protein extracts in 50-ml Falcon tubes before eluting the interacting proteins, which were resolved by SDS-PAGE cells, excised, and analyzed by matrix-assisted laser desorption/ionization peptide mass fingerprinting and tandem mass spectrometry (MS/MS), as detailed (Abenza et al., 2010).

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Pantazopoulos A, Peñalva MA (2009). Organization and dynamics of the Aspergillus nidulans Golgi during apical extension and mitosis. Mol Biol Cell 20, 4335–4347.

Pantazopoulos A, Peñalva MA (2011). Characterization of Aspergillus nidulans RabCoxes. Traffic 12, 386–406.

Peñalva MA (2005). Tracing the endocytic pathway of Aspergillus nidulans with FM4-64. Fungal Genet Biol 42, 963–975.

Peñalva MA (2010). Endocytosis in filamentous fungi: Cinderella gets her reward. Curr Opin Microbiol 13, 684–689.

Peplovska K, Markgraf DF, Ostrowicz CW, Bange G, Ungermann C (2007). The CORVET tethering complex interacts with the yeast Rab5 homolog Vps21 and is involved in endo-lysosomal biogenesis. Dev Cell 12, 739–750.

Pfeffer S, Alvezian D (2004). Targeting Rab GTPases to distinct membrane compartments. Nat Rev Mol Cell Biol 5, 886–896.

Petryaev D, Datta S, Ackema K, Zerial M, Spang A (2010). Identification of the switch in early-to-late endosome transition. Cell 141, 497–508.

Price A, Seals D, Wickner W, Ungermann C (2000). The docking stage of yeast vacuole fusion requires the transfer of proteins from a cis-SNARE complex to a Rab/Ypt protein. J Cell Biol 148, 1231–1238.

Rieder SE, Emr SD (1997). A novel RING finger protein complex essential for a late step in protein transport to the yeast vacuole. Mol Biol Cell 8, 2307–2327.

Rink J, Ghigo E, Kalaidzidis Y, Zerial M (2005). Rab conversion as a mechanism of progression from early to late endosomes. Cell 122, 735–749.

Rodríguez-Galán O, Galindo A, Hervás-Aguilar A, Arst HN Jr, Peñalva MA (2009). Physiological involvement in pH signalling of Vps24-mediated recruitment of Aspergillus PalB cysteine protease to ESCRT-III. J Biol Chem 284, 4404–4412.

Sánchez-Ferrero JC, Peñalva MA (2006). Endocytosis. In: The Aspergilli: Genomics, Medical Aspects, Biotechnology, and Research Methods, eds. GH Goldman, SA Osmani, Boca Raton, FL: CRC Press, 177–195.

Schuster M, Lipowsky R, Assmann MA, Lenz P, Steinberg G (2011). Transient binding of dynein controls bidirectional long-range motility of early endosomes. Proc Natl Acad Sci USA 108, 3618–3623.

Seals DF, Itzen G, Margolis N, Wickner WT, Price A (2000). A Ypt/Rab effector complex containing the Sec1 homolog Vps33p is required for homotypic vacuole fusion. Proc Natl Acad Sci USA 97, 9402–9407.

Segev N (2011). Coordination of intracellular transport steps by GTPases. Semin Cell Dev Biol 22, 33–38.

Steinberg G (2007). On the move: endosomes in fungal growth and pathogenicity. Nat Rev Microbiol 5, 309–316.

Szewczyk E, Nayak T, Oakley CE, Edgerton H, Xiong Y, Taheri-Talesh N, Osmani SA, Oakley BR (2006). Fusion PCR and gene targeting in Aspergillus nidulans. Nat Protoc 1, 3111–3120.

Taheri-Talesh N, Horio T, Araujo-Bazán L, Doux X, Espeso EA, Peñalva MA, Osmani SA, Oakley BR (2008). The tip growth apparatus of Aspergillus nidulans. Mol Biol Cell 19, 1439–1449.

Wedlich-Soldner R, Straube A, Friedrich MW, Steinberg G (2002). A balance of KIF1A-like kinesin and dynein organizes early endosomes in the fungus Ustilago maydis. EMBO J 21, 2946–2957.

Zekert N, Fischer R (2008). The Aspergillus nidulans kinesin-3 UncA motor moves vesicles along a subpopulation of microtubules. Mol Biol Cell 20, 673–684.

Zerial M, McBride H (2001). Rab proteins as membrane organizers. Nat Rev Mol Cell Biol 5, 107–117.

Zhang J, Yao X, Fischer L, Abenza JF, Penalva MA, Xiang X (2011). The p25 subunit of the dynactin complex is required for dynein-early endosome interaction. J Cell Biol 193, 1245–1255.

Zhang J, Zhuang L, Lee Y, Abenza JF, Peñalva MA, Xiang X (2010). The microtubule plus-end localization of Aspergillus dynein is important for dynein-early-endosome interaction but not for dynein ATPase activation. J Cell Sci 123, 3596–3604.