Transport of fungal RAB11 secretory vesicles involves myosin-5, dynein/dynactin/p25, and kinesin-1 and is independent of kinesin-3

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ABSTRACT Hyphal tip cells of the fungus Aspergillus nidulans are useful for studying long-range intracellular traffic. Post-Golgi secretory vesicles (SVs) containing the RAB11 orthologue RabE engage myosin-5 as well as plus end– and minus end–directed microtubule motors, providing an experimental system with which to investigate the interplay between microtubule and actin motors acting on the same cargo. By exploiting the fact that depolymerization of F-actin unleashes SVs focused at the apex by myosin-5 to microtubule-dependent motors, we establish that the minus end–directed transport of SVs requires the dynein/dynactin supercomplex. This minus end–directed transport is largely unaffected by genetic ablation of the Hook complex adapting early endosomes (EEs) to dynein but absolutely requires p25 in dynactin. Thus dynein recruitment to two different membranous cargoes, namely EEs and SVs, requires p25, highlighting the importance of the dynactin pointed-end complex to scaffold cargoes. Finally, by studying the behavior of SVs and EEs in null and rigor mutants of kinesin-3 and kinesin-1 (UncA and KinA, respectively), we demonstrate that KinA is the major kinesin mediating the anterograde transport of SVs. Therefore SVs arrive at the apex of A. nidulans by anterograde transport involving cooperation of kinesin-1 with myosin-5 and can move away from the apex powered by dynein.

INTRODUCTION Intracellular transport is crucial for eukaryotic cells. Cytoplasmic dynein (Cianfrocco et al., 2015) and kinesin(s) (Vale, 2003) move cargo toward the minus and plus ends of microtubules (MTs), respectively, whereas myosin-5 transports cargo toward the barbed ends of actin filaments (AFs; Reck-Peterson et al., 2000; Hammer and Sellers, 2012). Within the cell, the directionality of transport is dictated by the localization and orientation of cytoskeletal tracks. For example, the orientation of MTs radiating from perinuclear MT-organizing centers (MTOCs) in higher eukaryotic cells implies that plus end–directed kinesins normally mediate centrifugal transport, whereas cytoplasmic dynein mediates centripetal transport. Certain filamentous fungi, such as Ustilago maydis and Aspergillus nidulans, are genetically amenable and experimentally advantageous to study long-distance transport due to the large intracellular distances within their tubular cells, denoted hyphae (Peñalva et al., 2012; Steinberg, 2014; Xiang et al., 2015). In hyphal tip cells, which grow by apical extension, centrifugal and centripetal transport correspond to anterograde (apex-directed, acropetal) and retrograde (away from the apex, basipetal) transport, respectively.

In both U. maydis and A. nidulans, early endosomes (EEs) are the best-studied dynein cargo. EEs move by kinesin-3 toward the MT plus ends at the hyphal tip and by dynein away from the hyphal tip, toward MT minus ends associated with MTOCs embedded in the nuclear membranes (Wedlich-Soldner et al., 2002; Lenz et al., 2006; Abenza et al., 2009, 2010, 2012; Zekert and Fischer, 2009; Egan et al., 2012; Seidel et al., 2012, 2013). The physical interaction between dynein and EEs is mediated by the dynactin and

Monitoring Editor
Gero Steinberg
University of Exeter
Received: Aug 3, 2016
Revised: Jan 31, 2017
Accepted: Feb 7, 2017

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E16-08-0566) on February 16, 2017.
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Abbreviations used: AF, actin filament; EE, early endosome; latB, latrunculin B; MT, microtubule; MTOC, microtubule-organizing center; SPK, Spitzenkörper; SV, secretory vesicle; TGN, trans-Golgi network; wt, wild type.
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Vegetative hyphae of A. nidulans are well suited for study of the transport of secretory vesicles (SVs) that connect the trans-Golgi (TGN) with the plasma membrane (PM). These SVs arise from the TGN by maturation (Pantazopoulou et al., 2014; Pinar et al., 2015), which involves the recruitment to TGN cisternae of A. nidulans RabERAB11 and the subsequent engagement of the molecular motors that mediate the delivery of SVs to the apex, where they accumulate before undergoing fusion with the plasma membrane. As exocytosis supports apical extension, transport impairment slows down growth, besides altering morphology. A. nidulans exocytosis is an example of cooperation between actin and MT motors (Horio and Oakley, 2005; Taheri-Talesh et al., 2008, 2012; Zhang et al., 2011a; Pantazopoulou et al., 2014). Evidence supports a relay mechanism (Figure 1A) by which SVs are moved by kinesin(s) from apex-distal regions to the hyphal tip, where they are handed over to the MyoE myosin-5 for the final step of transport to the plasma membrane (Sharpless and Harris, 2002; Taheri-Talesh et al., 2011; Pantazopoulou et al., 2014). It is of note that SVs are loaded with kinesin(s) and with the single A. nidulans myosin-5, MyoE (Pantazopoulou et al., 2014). They appear to be also loaded with a minus end-directed motor, supposedly dynein. In the current model, if the relay between MTs and AFs fails, SVs move away from the tip toward the minus ends of MTs and then shift their direction to start another round of antero-grade transport by kinesin(s)/myosin-5 (Figure 1A).

Cooperation between kinesin(s), myosin-5, and, hypothetically, dynein in the transport of A. nidulans SVs is interesting for two reasons: one is that in this cooperation, the fungus resembles higher eukaryotes (Wu et al., 1998; Hammer and Sellers, 2012). The second and more important is that SVs are well suited to investigate how cargo is engaged simultaneously by different motors, which are the physiological consequences of physical interactions between kinesin and myosin-5 (Huang et al., 1999), and how one motor affects the other’s tracks when they coexist as passengers of the same cargo (Chesarone-Cataldo et al., 2011).

Here we demonstrate that SVs can indeed engage dynein. Although EEs and SVs move independently of each other, they share the requirement for the p25 subunit of dynactin to recruit the dynein supercomplex, suggesting that p25 might play a broad role in connecting several membranous cargoes to dyactin. However, EEs and SVs differ in two important features: one is that, unlike EEs, SVs do not require the Hook complex to engage dynein. The other is that, unlike EEs, which use kinesin-3 (UncA in A. nidulans) for their plus end-directed movement (Wedlich-Soldner et al., 2002; Zekert and Fischer, 2009; Schuster et al., 2011; Egan et al., 2012; Seidel et al., 2013), SVs use mainly, if not exclusively, the A. nidulans kinesin-1, KinA.

RESULTS
In the steady state, SVs labeled with RabERAB11 accumulate in the apex, forming a structure denoted Spitzenkörper (SPK; Figure 1A). Kinesin(s) and the MyoE myosin-5 cooperate to deliver SVs to the tip region (see later discussion), and yet the sharp focusing of SVs at the SPK is solely mediated by the myosin-5, exploiting the network of AFs that radiate from the apex (Sharpless and Harris, 2002; Pantazopoulou et al., 2014). They appear to be also loaded with a minus end-directed motor, supposedly dynein. In the current model, if the relay between MTs and AFs fails, SVs move away from the tip toward the minus ends of MTs and then shift their direction to start another round of antero-grade transport by kinesin(s)/myosin-5 (Figure 1A).

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FIGURE 1: Interplay between F-actin and MT motors in the transport of A. nidulans SVs: the effects of depolymerizing F-actin. (A) Kinesin moves RabERAB11-containing SVs (small green ovals) to the tip region, where they are handed over to myosin-5, which normally powers the AF-dependent final stage of exocytosis between the TGN cisternae and the apex. After arriving at the SPK (an apical accumulation of SVs), a minor proportion undergoes transport away from the apex. Latrunculin B treatment releases SVs localized by actomyosin in the SPK. These unleashed SVs undertake plus end– and minus end–directed conveyor belt–like movement using MT motors. (B) Top, delocalization of the SPK GFP-RabE SVs that results from AF depolymerization. Bottom, line scans derived using the regions indicated in blue and magenta for untreated and latB-treated wt cells, respectively. (C) Kymographs derived from the same hyphae and lines used for the line scans in B. The strong signal at the apex of the untreated cell corresponds to the SPK. Time resolution, 10 frames/s. (D) Maximal intensity projection of the time stack used in C for the wt cell treated with latB. (E) Average speeds of SVs derived from kymographs for 19 antero-grade and 20 retrograde SV movements pooled from three wt hyphae treated with latB at 28°C. Mean values were statistically significant (p < 0.0001) in an unpaired t test.

Taheri-Talesh et al., 2012; Pantazopoulou et al., 2014). Supplemen-tal Movie S1 illustrates the strong accumulation of SVs at the apex, using moving EEs as internal reference. EEs move on dynein and...
kinesin but, unlike SVs, do not engage MyoE and are uniformly distributed across hyphae.

Depolymerization of the tip network of AFs with the anti–F-actin drug latrunculin B (latB) rapidly dissipates the SPK, both by eliminating the MyoE-driven transport of SVs to the apex and by preventing the MyoE-independent tethering of SVs to AFs (Pantazopoulou et al., 2014; Figure 1, A and B). The two effects combined unleash SVs to MT-dependent motors, resulting in the plus end– and minus end–directed conveyor belt–like movement of SVs that is conveniently displayed by kymographs, in which traffic shows up as diagonal tracks whose slopes correspond to the speeds of individual runs (Figure 1C). Maximal intensity projections (MIPs) of time stacks of SVs in latB-treated hyphae indeed reveal the MT tracks (Figure 1D). Supplemental Movie S2 depicts a latB-treated hypha in which the latB conveyor–like movement of SVs and the long-distance transport of EEs were cofilmed. Comparison of Supplemental Movies S1 and S2 helps in appreciating the remarkable effect that depolymerizing F-actin causes on SVs.

To determine the speed at which SVs move in this latB conveyor belt, we drew kymographs covering the apicalmost 20 µm of hyphae and tracked SVs manually, using MetaMorph software. Figure 1E shows pooled data of anterograde and retrograde runs for latB-treated hyphae cultured at 28°C. (Here and throughout the text, we use the terms anterograde and retrograde to indicate movement toward or away from the tip, respectively, rather than the direction of traffic toward or away from the plasma membrane.) Retrograde runs were, on average, significantly faster than anterograde runs (2.6 ± 0.7 SD µm/s vs. 3.3 ± 0.7 SD µm/s; p < 0.0001 in an unpaired t test). In the tip region, MTs are oriented with their plus ends toward the apex (Han et al., 2001; Egan et al., 2012). Therefore, these data extend our previous observations indicating that SVs engage minus end–directed motors and plus end–directed kinesin(s) (Pantazopoulou et al., 2014).

Retrograde transport of RabERAB1 secretory vesicles: a dynine functional assay

To study the involvement of dynine in the retrograde movement of SVs, we determined with the latB conveyor belt assay the effects of heat-sensitive (ts) mutations affecting key components of the dynine pathway, namely the dynine heavy chain (HC) NudA (nudA2ts and nudA5ts), the dynine regulator NudFLIST (nudF7ts), and the dynactin constituent NudKlings (nudK317ts; see scheme of the dynine–dynactin complex in Figure 2A). The yeast orthologue of NudF binds the HC and prevents its detachment from MTs even during cycles of ATP hydrolysis (Huang et al., 2012; Toropova et al., 2014). In A. nidulans, and U. maydis, NudFLIST is required for initiation of dynine–mediated endosome movement (Lenz et al., 2006; Egan et al., 2012). ARP1 (NudK) is the major subunit of the actin–like minifilament within the dynactin complex (Chowdhury et al., 2015; Urnavicius et al., 2015), and thus its mutational inactivation is expected to result in a dynactin deficit. Dynactin binds to and collaborates with cargo adaptors to increase dynine processivity (Cianfrocco et al., 2015).

Strains carrying nudA2ts, nudA5ts, nudF7ts, and nudK317ts grow normally at 28°C but show characteristic nud mutant growth at 37°C (Supplemental Figure S1), indicating compromised function of the dynine complex. None of the mutants showed signs of disorganization or hypertrophy of the GFP–RabERAB1 SPK at 28°C or after a 45– to 90-min incubation at 37°C (Supplemental Figure S1). In wild-type (wt) cells shifted to 37°C, latB also delocalized SVs to the MT conveyor belt, as at 28°C. We acquired stacks of images over time and used them to draw kymographs covering the apicalmost 20 µm of hyphae. Tracking of runs captured by kymographs in wt cells treated with latB at 37°C revealed average speeds of 3.5 ± 0.9 SD and 4.5 ± 1.1 SD µm/s for anterograde and retrograde runs, respectively (Figure 2B). This significant difference observed between anterograde and retrograde speeds (p < 0.0001 in unpaired t test) is consistent with the notion that different MT motors mediate anterograde and retrograde runs. Of note, measured average speeds at 37°C were ~35% higher than those at 28°C (Figures 1E and 2B). Next we used latB to treat nud mutants that had been previously shifted to 37°C for 30–45 min, seeking to impair the motor’s function before unleashing SVs from the SPK. In the wt and in all four dynine pathway mutants, latB caused SV detachment from the apex. However, whereas SVs redistributed across hyphae more or less uniformly in the wt, they remained clustered in the tip regions in nudA2, nudA5, nudF7, and nudK317 strains (Figure 2, C–E), indicating that their retrograde transport requires dynine. Because the nudA2, nudA5, nudF7, and nudK317 alleles may differ in their severity and they affect proteins with different roles, any conclusion obtained by comparing mutant strains should be interpreted with caution. Despite this caveat, all four mutants showed a remarkably similar phenotype, presenting large aggregates of SVs within the ~5-µm most apical region of hyphae that were absent from the wt.

We acquired stacks of images over time of latB-treated mutants (time resolution, 400 frame/min, 100–300 frames each). Movies showed that the tip aggregates were subject to forces acting in the retrograde direction and suggested that even though motors attempted to pull the mutants’ aggregates away from the apex, they were incapable of dispersing them retrogradely, which is consistent with a dynine defect. A typical example is shown in Supplemental Movie S3, which is a composite showing a nudA5 movie alongside a control movie of the wt subject to the same experimental regime. Movies also showed that very few runs were visually noticeable in the mutants, an observation that was confirmed with kymographs (Figure 2E). In stark contrast with the numerous anterograde and retrograde trajectories of SVs detached from the SPK at 37°C in the wt, only a few trajectories were detectable in the mutants (Figure 2E), and these become visible only after overcontrasting the kymographs strongly. The observation that not only retrograde movements but also anterograde ones were very scarce in latB-treated nud cells strongly suggests that in the wt it is the pool of RabERAB1 SVs residing in the SPK (and not SVs generated de novo) that represents the major source of membranous structures moving on the latB conveyor belt. Indeed, inspection of nud hyphal tips immediately after addition of latB showed that the subapical clusters seen in the mutants are formed by detachment of SVs that had already accumulated at the SPK. Taken together, the preceding data led us to conclude that the blockade at the tip region of the latB–triggered retrograde transport of SPK SVs provides an easily scorable assay for dynine function.

Recruitment of the dynine complex to SVs requires the dynactin component p25 but not the EE adaptor HookA

EEs move on MTs. EEs are loaded with dynine within the tip region (the dynine loading zone; Lenz et al., 2006; Schuster et al., 2011). Engagement of EEs to dynine requires dynactin, specifically its p25 subunit (Zhang et al., 2011b; Figure 2A), and the Hook (FHF) complex adaptor (Biel ska et al., 2014; Yao et al., 2014; Zhang et al., 2014), which is an effector of GTPase RAB5 acting on the membrane of EEs (Guo et al., 2016). Accordingly, in p25Δ or hookAΔ cells, EEs remain stalled in the tip due to their inability to move in the retrograde direction (Biel ska et al., 2014; Yao et al., 2014; Zhang et al., 2014), as shown in the RabERAB1 channels of Supplemental Movies S4 and S5 and in Supplemental Figure S2. SVs gather at the SPK of p25Δ and hookAΔ hyphae in the wt (Figure 3A and Supplemental
Movies S4 and S5), as expected from the fact that the wt distribution of SVs mainly reflects anterograde transport by kinesin and myosin.

To test whether SVs have the same requirements as EEs to engage dynein, we used p25∆ and hookA∆ mutants in latB assays. Of note, we observed a stark difference between the two mutants. Contrary to EEs, which remained stalled at the mutant tips (Supplemental Figure S2 and Supplemental Movie S6), SVs of hookA∆ cells were shifted to the MT-dependent conveyor belt as in the wt (Figure 3, A and B). When taken together with experiments using dynein pathway mutants (Figure 2), this observation establishes that the dynein-dependent retrograde transport of SVs accumulated at the apex does not require the FHF adaptor complex. In contrast, SVs were detached from the apices of p25∆ cells by latB but did not undergo retrograde transport (Figure 3, A and B), remaining stalled in the tips, like EEs (Supplemental Figure S2 and Supplemental Movie S7). Thus the behavior of SVs in p25∆ cells resembles that of dynein pathway mutants. We concluded that dynein engagement by SVs requires dynactin p25 but not HookA.

To characterize in greater detail the effects of p25∆ and hookA∆ on the motility of SVs, we counted the number of RabE RAB11 vesicle runs per minute in samples of wt, p25∆, and hookA∆ cells treated with latB, using 20-µm-long kymographs starting at the apex (Figure 3B). This quantification yielded similar numbers of anterograde and retrograde runs per wt cell (mean values, ∼130 runs/min in each direction; Figure 3C). This traffic was abolished by p25∆ (Figure 3, B and C), as expected from the fact that most RabE RAB11-positive membranes were stalled in the p25∆ tips (Figure 3A). In marked contrast, hookA∆ cells were only moderately affected: the number of runs per cell was reduced by 50% (or even less in the case of anterograde movements) in the hookA∆ mutant compared with the wt (Figure 3C). Moreover, the speeds of anterograde and retrograde SV runs (2.2 ± 0.5 SD) traced across the hyphal axis of the cells in C. The same wt control is included in all panels for clarity. (E) Kymographs derived from 22.5-s movies (400 frames/min) using lines traced longitudinally across the 20-µm apicalmost regions of hyphae of the indicated strains. Colors used for the kymograph strain labels match those of the line scans in D. The table summarizes the number of SV movements (runs).
and 3.0 ± 1.0 SD µm/s, respectively; Figure 3D), were slightly reduced relative to the wt (see Figure 1E for the wt at 28°C). These moderate effects of hookΔ on the flux and speed of SVs and the fact that SVs are completely released from the apices by latB strongly argue against a direct involvement of HookA in retrograde transport. Moreover, the fact that in hookΔ cells, RabERAB11 SVs are transferred efficiently to MT-dependent motors rules out the possibility that it is the accumulation of EEs at the tip that actually precedes, due to MT traffic congestion, the dynein-dependent movement of RabERAB11 membranes in the latB-treated p25Δ mutant. Therefore we conclude that p25 of dynactin is absolutely required for the association of the dynein complex with both SVs (Figure 3E) and EEs, whereas the HookA/FHF complex adaptor is required for dynein engagement to EEs and dispensable for its engagement to SVs.

The retrograde transport of RabERAB11 SVs observed in latB-treated hookΔ cells establishes that SV motion does not involve hitchhiking on EEs, because EE movement requires HookA. This conclusion is important because in this particular regard, MT-dependent SV traffic differs from that of polysomes, peroxisomes, and lipid droplets, which distribute across hyphal cells by hitchhiking on EEs (Higuchi et al., 2014; Guimaraes et al., 2015; Salogiannis et al., 2016). It is also consistent with our previous observations that SVs do not colocalize with EEs labeled with RabAAB5 or FYVEVps27 (a phosphatidylinositol 3-phosphate–binding duplicated copy of the Vps27 FYVE domain; Panta-poulou et al., 2014). Finally, the requirement of p25 for the dynein-mediated transport of two different cargoes, EEs and SVs, suggests that p25 facilitates the interaction of dynactin with two different cargo adapters (the FHF complex for EEs and an as-yet-identified adaptor for SVs) and/or has a broad role in dynactin function (see the Discussion).

**kinΔ** impairs the retrograde movement of SVs away from the SPK by causing a dynein deficit at the tips

KinA is required for the transport of dynein to the plus ends of MTs (Zhang et al., 2003). Therefore **kinΔ** results in deficient supply of dynein to the hyphal tip, which leads to EEs accumulating in this region due to their inability to move away retrogradely (Lenz et al., 2006; Abenza et al., 2009). Similarly, SVs detached by latB from the SPK would be expected to accumulate in **kinΔ** mutant tips, resembling nudA, nudF, and nudKAPPI heat-sensitive as well as p25-null mutant tips. LatB treatment of **kinΔ** cells expressing GFP–RabERAB11 showed that this is indeed the case (Figure 4A). The penetrance of this phenotype (i.e., the formation of accretions of SVs in the tips of latB-treated **kinΔ** cells) was nearly complete: 90% (n = 80) of **kinΔ** tips contained SV aggregates, contrasting with only 6% (n = 124) of tips of similarly treated wt cells. However, inspection of **kinΔ** aggregates showed that they were generally less bright than those forming in dynein pathway mutants. We also observed this weaker-than-**nud** **kinΔ** phenotype with the tip aggregates of EEs (Abenza et al., 2009), which hinted at the possibility that the dynein deficit at the **kinΔ** tips might not be complete. To address this possibility, we introduced an endogenously tagged nudA-3xGFP allele (Egan et al., 2012) into the **kinΔ** mutant. In the wt, NudA associated with the plus ends of growing MTs, which results in comet-like structures that move toward the tip (Supplemental Movie S8), as
Evidence that KinA (kinesin-1) is the main kinesin cooperating with MyoE (myosin-5) in SV transport

A role for kinesin(s) in the anterograde transport of SVs was inferred from several observations (Pantazopoulou et al., 2014): 1) MT depolymerization markedly decreases the apical accumulation of SVs in the wt and completely prevents it in a myoEΔ mutant; 2) kymograph analyses revealed a class of SV anterograde trajectories moving over several micrometers with uniform speeds toward the tip; and 3) myoEΔ, albeit debilitating, does not impede growth, and indeed the presence of RabERAB11 SVs delivered by active transport (i.e., with speed and directionality incompatible with diffusive mechanisms) to the apical dome of myoEΔ cells has been documented (Pantazopoulou et al., 2014). We confirmed that this transport is actually MT mediated by coimaging GFP-RabERAB11 SVs with GFP-labeled MTs in myoEΔ cells (Figure 5A). Supplemental Movie S10 is a composite showing a GFP-MT cell alongside a GFP-MTs-and-GFP-SVs doubly labeled cell to illustrate that the fainter MTs do not interfere with the visual tracking of SVs and that the latter can be distinguished moving on MTs. Therefore, considering the orientation of tip MTs, the contribution of MT-mediated transport to SV delivery must involve kinesin(s). However, the identity of the kinesin(s) is currently unknown.

We approached this problem genetically, exploiting the phenotypes of null mutants in motor-encoding genes. Note that kinesin and myosin-5 activities cooperate to deliver SVs, but the motors do not strictly act in parallel. Kinesin(s) move vesicles toward the tip but cannot compensate for the absence of MyoE because MT transport cannot focus SVs at the growing apex (Taheri-Talesh et al., 2012; Pantazopoulou et al., 2014). Thus SV delivery is very inefficient in myoEΔ cells. In contrast, MyoE transport is expected to compensate for the absence of the kinesin(s) because actin cables radiating from the apex (and therefore the activity of MyoE motors) stretch backward into the subapical region in which TGN cisternae (the origin of SVs) are highly abundant (Pantazopoulou and Peñalva, 2009), making the contribution of kinesin relatively less important. In such a scenario, combining SV kinesin and myosin-5 null mutations should preclude growth, leading to lethality. A clear candidate for an SV kinesin is KinA because a kinA disruption allele (kinA::pyr4; Requena et al., 2001; 2014) is virtually lethal when combined with MyoE (myosin-5) down-regulation (Zhang et al., 2011a). Given the importance of this observation, we constructed complete deletion alleles of kinAΔ (removing kinesin-1) and uncAΔ (removing the endosomal UncA kinesin-3; Zekert and Fischer, 2009) to revisit their genetic interactions with a null myoEΔ allele (Taheri-Talesh et al., 2012).

We first studied the effects of the mutations in colony growth on solid medium. Compared to the strong myoEΔ colony growth defect, kinAΔ and uncAΔ single-deletion mutations had a minor negative effect on growth, although that of kinAΔ was more

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We first studied the effects of the mutations in colony growth on solid medium. Compared to the strong myoEΔ colony growth defect, kinAΔ and uncAΔ single-deletion mutations had a minor negative effect on growth, although that of kinAΔ was more
negative additivity, leading to barely visible colonies that could not be further subcultured (arrowed on Figure 5C). Combining myoEΔ with kipAΔ (removing the kinesin KipA involved in growth directionality determination; Konzack et al., 2005) did not have any effect (unpublished data). Therefore genetic data strongly suggest that KinA is the major kinesin cooperating with MyoE to deliver SVs to the apex. To estimate quantitatively the contributions of motors to exocytosis, we determined by time-lapse microscopy actual apical extension rates of individual wt and mutant tip cells cultured in minimal medium, using inverted microscope chambers incubated at 29.5 ± 0.5°C. Under these conditions, wt cells grew at 1.01 ± 0.2 µm/min (mean ± SD), whereas uncAΔ cells grew significantly more slowly than the wt, at 0.8 ± 0.17 µm/min (~20% reduction; Figure 5D). The kinAΔ tips grew even more slowly (at 0.66 ± 0.13 µm/min, ~35% reduction), and myoEΔ, which shows a strong colony growth defect (Figure 5, B and C; Taheri-Talesh et al., 2012), reduced the actual apical extension rate by ~75% (to 0.26 ± 0.1 µm/min; Figure 5D). Thus these differences roughly resemble those observed in colony growth tests. However, these assays could not discriminate the minor differences in (poor) growth detected by colony tests between single myoEΔ and double myoEΔ uncAΔ cells.

Attempting to correlate the apical extension rate defects with exocytic traffic impairment, we tested by time-lapse imaging whether the absence of either KinA or UncA affected the steady-state accumulation of RabERAB11 SVs at the SPK. Neither uncAΔ nor kinAΔ impeded such accumulation. However, we noticed both reduced compactness and apparently reduced brightness specifically in the SPK of kinAΔ hyphae. Prompted by these observations, we determined the fluorescence intensity of GFP-RabERAB11 in the SPKs of the wt and kinesin mutant strains (Figure 5E), which confirmed that the kinAΔ SPK shows half the fluorescence of the wt, whereas the uncAΔ SPK was much less, yet significantly affected, in agreement with colony growth and apical extension rate tests. The fact that the double kinAΔ uncAΔ mutant was not significantly different from the single kinAΔ mutant (Figure 5E) further indicates that KinA plays the major role in the transport of SVs.

FIGURE 5: Genetic data strongly suggest that kinesin-1 (KinA) cooperates with myosin-5 (MyoE) in exocytosis. (A) myoEΔ hyphal tip cells expressing GFP-α-tubulin alone (as control) or coexpressing GFP-α-tubulin and GFP-RabERAB11 to detect MTs and SVs, respectively. The double-labeled cell image is overcontrasted to show SVs aligned along MTs. Images were treated with the unsharp filter of MetaMorph. (B) Colony-growth phenotypes of motor mutant strains at 37°C. Colonies of indicated genotypes were ordered from left to right according to the growth impairment caused by the single- or double-null alleles. Note that all mutant motor strains carry yA2 resulting in yellow conidiospores (wt color is green) and that uncAΔ and myoEΔ show a weak, yet noticeable synthetic negative interaction. (C) A double myoEΔ kinAΔ mutation is virtually lethal. Left, diagnostic PCRs used to genotype deletion alleles. Right, progeny of a heterozygous parental strains plated on rich medium and incubated at 37°C. The minute colonies indicated with white arrows were diagnosed as myoEΔ kinAΔ double mutants by PCR. A and E indicate kinAΔ and myoEΔ progeny, respectively; wt is kinAΔ+ myoEΔ+ (yellow or green conidiospores). (D) Quantitation of apical extension rates in the wt and indicated single- and double-mutant strains. Strains were cultured at 29.5 ± 0.5°C on the microscope stage before collection of time stacks using Nomarski optics, which were used to deduce apical extension rates of individual cells. Scatter plots show mean ± 95% CI. The different data sets were compared by ANOVA followed by Bonferroni’s multiple comparison posttest. ***p < 0.001; ns, nonsignificant.

Rigor kinesin mutants reveal a direct role of KinA in the anterograde transport of RabERAB11 SVs

A potential drawback of the foregoing experiments is that in view of its numerous physiological roles in MT transport, ablation of
kinesin-1 might affect growth indirectly, as illustrated by the described kinAΔ defect on dynein localization. For example, kinA disruption stabilizes MTs (Requena et al., 2001). More-stable MTs would not affect transport per se, but MT and actin transport act in concert (the tip growth apparatus; Taheri-Talesh et al., 2008), and thus a hyperstable MT network could affect the network of AFs converging at the apex. However, the MyoE steady-state localization to the apex, dependent on this network, is not altered by kinAΔ (Supplemental Figure S3), arguing strongly against this possibility. Another possibility would be that given that U. maydis kinesin-1 is required for efficient MT bundling (Straube et al., 2006), kinAΔ weakens MT bundles at the tip, creating a gap between MTs and the cortex that debilitates MT-mediated exocytosis, making MyoE essential. This possibility was discarded after observing, in kinAΔ tips, GFP-labeled MTs, which are clearly capable of reaching the apical dome (Supplemental Figure S4).

In view of the foregoing considerations, we sought evidence supporting a direct role of KinA in SV transport. We used gene replacement to construct mutant alleles encoding UncAG116E and KinAG97E rigor kinesins (Zekert and Fischer, 2009). These substitutions in the respective Walker motifs do not preclude MT binding but disable motor activities due to deficient ATP cycles. First, we characterized the efficacy of the rigor mutations by imaging the behavior of EEs labeled with mCherry-RabA RAB5 by time-lapse microscopy. In the wt, EEs show an even distribution across hyphae (Figure 6A) and display characteristic anterograde and retrograde movements powered by kinesins (Abenza et al., 2009; Zekert and Fischer, 2009; Zhang et al., 2011b, 2014; Egan et al., 2012; Yao et al., 2014, 2015; Xiang et al., 2015). These movements can be captured by kymographs (Figure 6B). We deduced from these kymographs the average anterograde and retrograde speeds of EEs in the wt, which were 2.7 ± 0.6 SD and 3.3 ± 0.8 SD µm/s, respectively (Figure 6C). This significant difference (p < 0.001 in unpaired t test) had not been detected in previous works (including our own; Abenza et al., 2009; Egan et al., 2012), which is possibly related to improvements in the in vivo imaging of mCherry-RabA RAB5 (see Materials and Methods). Given the polarity of MTs in the tip-proximal region, these data suggest that dynein may be more efficient than kinesin-3 in the overall speed output at which motor teams propel EE movement. Physiological speeds reflect the influence of numerous factors besides the motors’...
biochemical activity, and these factors could include the balance between opposing motor teams binding to the same organelle. Of note, in U. maydis, a single dynein dimer can support retrograde EE transport, whereas anterograde movement requires several kinesin motors: examples and line scans. The yellow arrowhead points at a MT decorated with rigorously bound SVs in the kinAG97E hypha. (B) Wide-field sections derived from a z-stack of images of a kinAG97E cell showing GFP-RabERAB11 SVs decorating MTs (indicated with arrows). Z-step, 0.25 µm. (C) Anterograde runs attributable to kinesin-1 in subapical regions of hyphae. Time stacks of images of wt and mutant hyphae were used to construct kymographs. To minimize the effect of anterograde AF-mediated transport, these kymographs were drawn to detect the flux of vesicles across 10-µm-long apex-proximal regions that excluded the 2- to 4-µm apicalmost areas of hyphae. Faint anterograde runs, attributable to MT transport, were conspicuous in wt and uncaG116E but not in kinAG97E hyphae. (D) Flux of anterogradely and retrogradely moving SVs in regions described in C in eight hyphae for each genotype. The total number of runs per hyphae and minute was determined by visual inspection of kymographs. Runs were classified as anterograde or retrograde and plotted as means with 95% CIs. The different data sets were compared by ANOVA followed by Bonferroni’s multiple comparison posttest. ***p < 0.001, *p < 0.05; n.s., nonsignificant.

Kymographs were also used to estimate EE motility in any given cell as the total distance covered by EE runs per unit time (Figure 6D). Both uncaG116E and KinAG97E affected the motility of EEs markedly (Figure 6, B and D). In uncaG116E cells, EEs were uniformly distributed along the hyphae as in the wt (Figure 6A, line scans), but in sharp contrast with the latter, they were completely immotile (Figure 6, B and D), in agreement with reports that UncA is the principal kinesin powering EEs (Wedlich-Soldner et al., 2002; Zekert and Fischer, 2009; Egan et al., 2012). In contrast, KinAG97E cells were largely devoid of EE movement (Figure 6, B and D) because EEs accumulated in the tip (Figure 6A, yellow arrowhead), indicating that EEs arrive normally at this region (KinA does not move EEs) but are inefficiently transported retrogradely due to local dynein deficit resulting from kinAG97E. These dynamic phenotypes are better displayed as movies. Supplemental Movie S11 shows the almost complete absence of EE movement resulting from uncaG116E, and Supplemental Movie S12 shows that kinAG97E EEs accumulate in the slightly subapical dynein-loading zone (Lenz et al., 2006). We concluded that the kinesin alleles behaved as expected for rigor mutations.

We next studied the localization of RabERAB11 SVs in hyphae expressing UncAG116E or KinAG97E, which was essentially wt, that is, apical accumulation of SVs with some labeling associated with TGN puncta (Figure 7A). However, inspection of time-lapse movies (Supplemental Movie S13 compares the wt and KinAG97E hyphae) suggested that the KinAG97E SPK was less compact. In addition, KinAG97E hyphae displayed two clear phenotypes with regard to SV transport. The first was that immotile SVs often decorated MTs in a beads-on-a-string manner. Supplemental Movie S14 (Figure 7A) shows the behavior over time of these rigorously bound SVs. Figure 7B shows consecutive sections of a z-stack showing MT tracks decorated with rigorously bound SVs all along a hyphal tip cell. These data strongly indicate that KinAG97E motors bind to SVs and that these motors loaded with SVs are able to bind MTs but are unable to move. The second phenotype was revealed after tracking movement of SVs with kymographs. It is important to note that both kinesin(s) and MyoE cooperate in the anterograde transport of SVs to the SPK, and thus, in cells that have not been treated with latB to remove the MyoE component of transport, anterograde movements detected by kymographs can be mediated by either MT or actin motors, which hampers quantitative analyses of MT transport. Attempting to reduce the relative weight of actin transport, we estimated the flux of vesicles across a linear 10-µm region that excluded the tip (i.e., 2- to 4-µm region proximal to the apex). In addition, by eliminating the highly fluorescent SPK region from our analyses, we improved detection of the faint runs that we attribute to MT-mediated events (Pantazopoulou et al., 2014). Kymographs of wt and uncaG116E cells indeed revealed faint anterograde RabERAB11 runs of similar and uniform speeds in these subapical regions, consistent with kinesin-mediated transport (Figure 7C). By counting these runs, we determined that the flux of anterograde SVs that move across 10-µm-long subapical regions was 55 runs/min in the wt. This flux...
The foregoing observations imply that UncA moves EEs (but not SVs), whereas KinA powers the anterograde transport of SVs toward the tip. Our experiments revealing weak effects of uncAΔ in apical extension and SPK brightness (Figure 5, D and E) suggested that UncA might play an ancillary role in anterograde SV transport. However, the finding that uncAΔG116E did not reduce the anterograde transport of SVs (Figure 7D) argues strongly against this possibility. Thus the foregoing uncAΔ phenotypes are perhaps attributable to an overall defect in EE transport resulting from uncAΔ. Moreover, if kinesin-1 (KinA) is the only MT motor mediating anterograde SV transport, the myoEΔ kinAΔG97E combination should be as debilitating as the nearly lethal myoEΔ kinAΔ combination. Indeed, the progeny of a heterozygous cross germinated in microscopy wells contained ascospores that gave rise to morphologically abnormal abortive germlings resembling those seen when myoE was down-regulated in a kinAΔ background (Supplemental Figure S5; Zhang et al., 2011a). Hence the genetic interaction between KinA and MyoE involves the cargo-powering role of the kinesin. Taken collectively, all the foregoing data strongly indicate that KinA is the principal kinesin cooperating with MyoE in the anterograde transport of SVs.

F-actin depolymerization shifts RabERAB11 SVs to MTs decorated by rigor KinAΔG97E. Rigor MTs decorated with SVs in KinAΔG97E hyphae abounded in regions located far from the apex but were sparser near the tips. This suggested that their detection in tip-proximal regions, in which actin transport is vigorous, might be hindered by the activity of MyoE, which could possibly snatch SVs rigorously bound to MTs and deliver them to the apex. Moreover, because in the steady state, the majority of SVs concentrate in the SPK due to myosin-5 transport, we hypothesized that MT decoration by rigor SVs would be markedly more abundant after releasing the SPK SVs from actin. To test this prediction, we treated cells with latB. Control experiments using EEs labeled with mCh-RabARAB5 established that EEs moved bidirectionally in the wt, were immobile in the UncA ΔG116E mutant, and accumulated in tips in the KinAΔG97E mutant (Supplemental Figure S6), as in untreated cells. We next focused on SVs. As in the wt, in both KinAΔG97E and uncAΔG116E cells, latB dissipated the SPK accumulation of SVs, which redistributed across hyphae (Figure 8A). In uncAΔG116E cells, SVs underwent bidirectional movements similarly to those in the wt (Figure 8B). Indeed, when we determined the number of vesicle

FIGURE 8: SVs rigorously bound by KinAΔG97E massively decorate MTs after latrunculin B treatment. (A) Localization of SVs labeled with GFP-RabERAB11 in the wt and in cells expressing rigor mutant kinesins with and without latB treatment. (B) Kymographs (10 µm long, starting at the apex) derived from time stacks of GFP-RabERAB11 images obtained from kinAΔG97E and uncAΔG116E cells treated with latB (600 frames/min). (C) Flux of anterograde and retrograde runs in regions described in B in five latB-treated hyphae for each genotype. The total number of runs per hypha and minute was determined by manual tracking on kymographs. Runs were classified as anterograde or retrograde and plotted as means with 95% CIs. The different data sets were compared by ANOVA followed by Tukey’s multiple comparison posttest. ***p < 0.001; n.s., nonsignificant. (D) KinAΔG97E cells coexpressing mCh-RabARAB5 to label EEs and GFP-RabERAB11 to label SVs. In the untreated KinAΔG97E control, SVs accumulate in the SPK and EEs in a slightly subapical aggregate. LatB treatment of KinAΔG97E cells shifts SVs from the SPK to MTs, which are conspicuously decorated with rigorously bound vesicles. Note that the tip accumulation of EE is not dispersed by actin depolymerization (see also Supplemental Movies S15 and S16).
runs per minute captured by kymographs in the apicalmost 20 µm of uncA<sup>G116E</sup> hyphae, we counted 162 ± 11 anterograde and 156 ± 14 (mean ± SE) retrograde runs per cell (Figure 8C). These figures are similar to those noted for the wt (Figure 3C). The high motility displayed by SVs in uncA<sup>G116E</sup> cells contrasts sharply with the complete absence of EE motility caused by this mutation (Figure 6, B and D; Supplemental Figure S6 and Supplemental Movie S14), implying that UncA moves EEs but not SVs.

The behavior of SVs in kinA<sup>G97E</sup> cells treated with latB was remarkable. kinA<sup>G97E</sup> prevented SV movement, reducing the number of SV runs per cell to almost negligible values (<15 anterograde or retrograde runs per minute per cell) compared with uncA<sup>G116E</sup> (and to the wt; see earlier discussion; Figure 8, B and C). This occurred because in kinA<sup>G97E</sup> cells, SVs released by latB were sequestered by MTs, which were very conspicuously decorated with vesicles, such that within <30 min after addition of latB, essentially all of the RabERAB11<sup>+</sup> fluorescence was seen on MTs (Figure 8D). Therefore the inability of RabERAB11SVs to move cannot result from their incapacity to productively meet and engage rigor KinA<sup>G97E</sup>. In shear contrast, EEs accumulate in the tips of this mutant (Figure 8D) and do not decorate MTs. Supplemental Movie S15 further shows that KinA<sup>G97E</sup> SVs do not move, as expected if they were attached to MTs by means of the rigor kinesin. Such inability is unlikely to result from a traffic jam caused by the KinA<sup>G97E</sup> rigor kinesin, as UncA<sup>G116E</sup> did not cause the same effect.

Supplemental Movie S16 visually summarizes the effects of latB on SVs in kinA<sup>G97E</sup>, uncA<sup>G116E</sup>, and wt cells. These effects and the genetic analyses in Figure 5 strongly support the contention that RabERAB11<sup>+</sup> SVs are loaded mostly, if not exclusively, with KinA, strongly indicating that it is this kinesin-1 that mediates the MT-dependent component of their anterograde transport.

**DISCUSSION**

Many subcellular membranous structures move on MTs and AFs. It is of general interest to understand the mechanisms by which kinesin, myosin-5, and dynein acting on the same cargo coordinate their action, as well the molecular codes by which motors discriminate their physiological cargoes among the lavish diversity of compositionally different intracellular membranous structures (Akhmanova and Hammer, 2010; Fu and Holzbaur, 2014).

Here we studied the long-range transport of A. nidulans RabERAB11<sup>+</sup> SVs to the hyphal apex, which involves kinesin and myosin-5 activities (Pantazopoulou et al., 2014). We establish that kinesin-1 (KinA) is the principal and perhaps the only kinesin powering the MT component of anterograde SV transport, whereas kinesin-3 (UncA) does not play an important role. Thus, according to the current model, SVs would move from tip-distal to tip-proximal regions with MTs/kinesin-1 and would be transferred to AFs/myosin-5 as they arrive to the proximity of the apex. Motor switching at the intersection between actin and MT tracks depends on which set of motors generates the greatest force (Schroeder et al., 2010). We speculate that SVs switch from kinesin-1 to myosin-5 as they reach the region where the AF network originating at the apex becomes denser, thereby increasing the overall performance of myosin-5 motors. Interplay between myosin-5 and kinesin-1 in exocytic transport is evolutionarily conserved. The nonmotile Saccharomyces cerevisiae kinesin-1–like protein Smy1 interacts with, and improves the processivity of, myosin-5 acting on SVs by tethering cargo to actin bundles (Hodges et al., 2009). Smy1 transported with SVs by myosin-5 to the sites of exocytosis regulates actin cable stability and dynamics (Chesarone-Cataldo et al., 2011) and, strikingly, Smy1 additionally enhances the association of yeast myosin-5 with SVs by reinforcing the interaction of the motor with the exocytic RAB Sec 4 (Lwin et al., 2016).

It is worth noting that besides powering the final stage of transport, MyoE<sup>Fyp53</sup> also focuses SVs at the apex. Therefore the MT and actin pathways do not act in parallel, as kinesin-1–based transport cannot perform the focusing step, explaining why myoE<sup>∆</sup> results in markedly defective apical extension, even though kinesin-1 can support exocytosis by way of the MTs that contact the apical dome. Our model also explains why kinesin-1 SV transport is much less important than MyoE transport: a large proportion of the otherwise strongly polarized TGN cisternae fall within the distance covered by actin cables and thus within the domain of action of MyoE. Our findings are also consistent with the report that KinA is involved in the transport of chitin synthases to the hyphal tip (Takehita et al., 2015). A recent landmark study with U. maydis characterized a class of secretory vesicles codelivering cell wall–synthesizing enzymes to the plasma membrane that is dependent on kinesin-1 and myosin-5 (Schuster et al., 2016).

Cytoplasmic dynein consisting of a dynein motor complex and a cofactor dynactin complex is an ~2.5-MDa minus-end MT motor of remarkable complexity (Chowdhury et al., 2015; Cianfrocco et al., 2015; Urnavicius et al., 2015). We established beyond doubt that A. nidulans SVs engage dynein/dynactin, as previously suggested by the finding that a proportion of them undergo retrograde movement once they arrive at the SPK (Pantazopoulou et al., 2014). This retrograde movement is conspicuously uncovered after removing with latB the paramount masking activity of AF-based anterograde transport/capture of SVs. We exploited this feature to demonstrate that the retrograde transport of SVs indeed depends on the dynein motor heavy chain, the dynein regulator NudF<sup>G151E</sup>, and the integrity of the dynein adaptor dynactin. The mechanisms by which dynein specifically recognizes its numerous membranous cargoes are being intensively studied. Many cargos exploit RAB effectors acting as adaptors to recruit the motor, with the RAB determining membrane specificity. For example, Golgi cisternae use RAB6 and its effector Bicaudal to bind dynactin (Hoogenraad et al., 2001; Urnavicius et al., 2015), perinuclear recycling endosomes use Rab11 and its effector FIP3 to bind the dynein light intermediate chain (Horgan et al., 2010), and late endosomes use Rab7 and its effectors RILP and ORP1L to bind p150<sup>Glued<sub>dynein</sub></sup> in dynactin (Johansson et al., 2007) and, in the case of RILP, also the dynein light intermediate chain (Scherer et al., 2014). The pointed-end complex capping the dynactin filament consists of Arp11, p62, p27, and p25 (Eckley et al., 1999) is accessible in the dynein–dynactin structure to interact with cargo adaptors without interfering with the motor’s activity (Chowdhury et al., 2015; Urnavicius et al., 2015), contributing a cargo entry site alternative to p150<sup>Glued<sub>dynein</sub></sup>.

Studies with U. maydis and A. nidulans led to the identification of the FTS/Hook/FHIP (FHF) complex on endosomes and of p25 in the pointed-end complex of dynactin as key players in the recruitment of dynein by EEs (Zhang et al., 2011b, 2014; Bielska et al., 2014; Yao et al., 2014; Xiang et al., 2015). The role of Hook proteins in dynein engagement was inferred from the accumulation of EEs unable to move retrogradely in the tips of cells carrying null hook mutations. Our studies with mutations affecting the dynein pathway showed that dynein inactivation in cells devoid of AFs led to the accumulation of SVs stalled in the tip region, providing a scorable assay for dynein function similar to that based on EEs. We demonstrate here that A. nidulans SVs do not have the same requirements as EEs to engage dynein: both fail to engage dynein in p25Δ cells, but SVs, unlike EEs, do not require HookA. The EE specificity of the Hook/FTH complex has been clarified by the finding that a complex
containing Hook1/3 and FHIP behaves as a RAB5 effector (Guo et al., 2016). Of note, the lack of Hook involvement in RabERAB11 SV transport was not predictive of a priori. Although the three mammalian Hook paralogues have been associated with endosomes (Xu et al., 2008; Maldonado-Báez et al., 2013), Hook3 reportedly localizes to the Golgi (Walenta et al., 2001), and Hook2 participates in the morphogenesis of the primary cilium (Baron Gaillard et al., 2011), a process that is regulated by a RAB cascade involving the RabE orthologue RAB11 (Das and Guo, 2011; Vetter et al., 2015).

Both SVs and EEs require p25 to engage dynein. Because dynein lacking p25 or p27 is stable (Zhang et al., 2011b; Yeh et al., 2012), EE and SV trafficking defects seen in p25-null mutant cells appear to reflect a broad role of p25 in cargo recognition rather than dynein disorganization. This agrees with the contention that p25 is conveniently positioned within the dynein–dyactin structure to mediate direct binding to different cargoes (Yeh et al., 2012). However, rather than directly bridging dynein to membranes, the p25-mediated interaction between dynein–dyactin and A. nidulans EEs necessitates HookA (Zhang et al., 2014), predictably recruited to EEs by Rab55s, as demonstrated for neurons (Guo et al., 2016). This suggests that there exists an as-yet-unidentified RabERAB11 effector acting as dynein adaptor, collaborating with p25 in the engagement of SVs, and, potentially, facilitating dynein processivity (McKenney et al., 2014; Olenick et al., 2016; Schroeder and Vale, 2016). Of note, sequence searches could not identify A. nidulans homologues of the coiled-coil–containing prototypic mammalian RAB11 effector and dynein adaptor FIP3 (Horgan et al., 2010).

In addition to dynein pathway mutants, ablation of kinesin-1 results in a dynein deficit at the tip (Zhang et al., 2003; Lenz et al., 2006) and impairs the retrograde movement of SVs (this work). Kine-
sin-mediated dynein localization at the microtubule plus ends is conserved from fungi to neurons (Zhang et al., 2003; Roberts et al., 2014; Twelvetrees et al., 2016). Our data, however, show markedly decreased yet detectable accumulation of dynein in the plus ends of MTS of kinAΔ tips, indicating that kinesin-independent mechanisms for dynein’s plus-end accumulation, such as direct recruitment from the cytoplasm, may be operative (Zhang et al., 2003; Markus et al., 2011; Cianfrocco et al., 2015). In this regard, dynein might also hitchhike on anterogradely moving RabERAB11 SVs to reach the tip in a myosin-dependent (thus kinesin–1-independent) manner.

As to why dynein engages and subsequently moves SV membranes retrogradely away from their normal destination, we can only speculate. One possibility is that the deposition of septa requires kinesin-1 and myosin-5 fails, SVs may move retrogradely before having a second chance of productively returning to the apex using anterograde motors (Pantazopoulou et al., 2014).

Finally, we point out that this relay model by which transport of RabERAB11 SVs depends on the anterograde (centrifugal) action of kinesin-1 and myosin-5 motors opposing the retrograde (centripetal) action of dynein resembles that proposed for melanophore transport (Wu et al., 1998), although in the latter, it is kinesin-2 rather than kinesin-1 that mediates the plus end–directed MT-dependent step (Levi et al., 2006; Kural et al., 2007).

MATERIALS AND METHODS

Aspergillus techniques and strains

Complete (MCA) and synthetic complete (SC) media (Cove, 1966) containing 1% glucose and 5 mM ammonium tartrate as carbon and nitrogen source, respectively, were routinely employed, with the excep-
tion of microscopy cultures (see later description). The complete genotypes of strains used for this work are listed in Supplemental Table S1. The rigor condition of KinA<sup>G97E</sup> and UncA<sup>G116E</sup> mutant ki-
esins was described previously (Zekert and Fischer, 2009). Site-di-
rected mutagenesis of kinA and uncA was used to generate mutant genes encoding KinA<sup>G97E</sup> and UncA<sup>G116E</sup> (the latter S-tagged in the C-terminus). This was followed by gene replacement after transformation with appropriate DNA fragments assembled by fusion PCR (Szewczyk et al., 2006). Transformation used Aspergillus fumigatus pygG (pyg<sup>G</sup>) as selective marker. The recipient strain was RQ54 (pyg<sup>G89</sup>; wA2; argB2::[argB*-alcAp::mCherry-RabA]; nkuAΔ::argB pyroA4; Qiu et al., 2013; nkuAΔ prevents nonhomologous recombi-
nation; Nayak et al., 2005). The resulting kinA<sup>G97E</sup> and uncA<sup>G116E</sup> mutant alleles were verified by DNA sequencing after PCR amplifi-
cation of the genes from genomic DNA. Deletion alleles of kinA (AN5343), uncA (AN7547), and kipA (AN8286) were made by substi-
tuting their complete coding regions by A. fumigatus pyrG or riboB, as appropriate (Supplemental Table S1).

DNA extraction for PCR genotyping of microcolonies

Entire microcolonies growing from ascospores were dispersed in 100 µl of 10 mM Tris-HCl, pH 8, 2% (vol/vol) Triton X-100, 1% (wt/ vol) SDS, 0.1 M NaCl, and 1 mM EDTA and disrupted by incubation at 70°C over a 30-min period with frequent vortexing. Proteins were removed by a single extraction with phenol/chloroform/isooamyl al-
cohol (25:24:1), and 0.5–1 µl of the recovered aqueous phase was used as DNA template in diagnostic PCRs.

General microscopy techniques and image acquisition

Unless otherwise indicated, epifluorescence microscopy was carried out with hyphae cultured in pH 6.8 watch minimal medium (WMM) at 28°C, using eight-well chambers (IBIDI, Martinsried, Germany, or Lab-Tek, Nalgé Nunc International, Rochester, NY) and a Leica DMi6000 B inverted microscope equipped with a Leica 63×/1.4 nu-
merical aperture Plan Apochromatic objective. The temperature of the chamber was controlled with a Heating Insert P on-stage incuba-
tor (Leica) and an objective heater (PeCon, Germany), as described (Pinar et al., 2013). Culture temperature in experiments with heat-
sensitive mutations was monitored within the wells using a wire ther-
ometer. Cultures reached 37°C within 15–20 min after the shift (Pinar et al., 2013). Latrunculin B was used at a final concentration of 0.1 mM, a concentration previously shown to completely depoly-
merize F-actin of endocytic patches within 2 min (Pantazopoulou and Peñalva, 2009).

The microscope, driven by MetaMorph Premier software (Mole-
cular Dynamics), was equipped with a Leica EL6000 excitation source, which was set to the minimal intensity compatible with ex-
posure times ranging from 100–250 ms to image the fluorescent proteins used in this work, except in the case of NudA-GFP<sup>x3</sup>, which required the maximal intensity of the excitation lamp com-
bined with exposure times of up to 600 ms when imaged in kinAΔ hyphae. Fluorescence images were captured with a Hamamatsu ORCA ER digital camera (1344 × 1024 pixels) using, for single-
channel acquisition, Semrock GFP—3035B and TXRED-4040B BrightLine filter cubes. For simultaneous channel acquisition, we used a Dual-View beam splitter (Photometrics) equipped with the supplier’s filter sets for GFP and mCherry fluorescence channels.
To optimize time resolution, movies were acquired using the streaming function of the software, and the smallest possible region of interest was selected to reduce the time needed to discharge the interline charge-coupled device (CCD) light sensor to the computer RAM.

Fluorescent proteins
GFP-RabERAB11 is an N-terminally tagged version of the exocytic RAB expressed under the control of its own promoter (Pantazopoulou et al., 2014). mCherry-RabARAB5 expression was driven by a single-copy integration transgene controlled by the alcA (alcohol dehydrogenase) gene promoter. α-Tubulin-GFP (TubA-GFP) was expressed from a single copy of the transgene integrated at the wA (white) locus. myoEΔ and wA::tubA-GFP strains used as parental strains for subsequent crosses were a generous gift of Berl Oakley (University of Kansas, Lawrence, KS). The parental nudA-GFPx3 strain used to study dynein in the kinAΔ background was a generous gift from Samara Beck-Peterson (University of California, San Diego).

To drive mCherry-RabARAB5 expression, we used our single-copy transgene based on the alcAp promoter (Abenza et al., 2009). alcAp is induced by ethanol and repressed by glucose. Overexpression of mCherry-RabARAB5 leads to detection of aggregates of EEs, presumably resulting from homotypic fusion of endosomes promoted by the excessive levels of RAB5. To prevent overexpression, we used D-fructose as C source. This noninduced and nonpressuring sugar result in levels of expression that resemble the physiological levels of RabARAB5 (Abenza et al., 2009). Using a 0.05% (wt/vol) concentration of fructose in overnight cultures, we achieved very homogeneous levels of induction in all hyphae of the population, with very little aggregation of EEs, which markedly facilitated the tracking of EE trajectories with kymographs traced across middle planes of individual hyphae. These kymographs were used to measure the speed of EEs at 28°C using the MetaMorph plug-in.

Image manipulation
All images and time series were processed using different plug-ins of MetaMorph 7.7.0, converted to 8-bit grayscale or 24-bit RGB and annotated with Corel Draw (Corel, Ottawa, Canada). When appropriate, images were corrected with the MetaMorph unsharp mask filter, using a kernel size of 6–9 and a scaling factor of 0.75. Precise alignment of Dual View channels was carried out using the color align menu of MetaMorph and distinctive features in the images as internal references or, when needed, fluorescent beads (TetraSpeck microspheres, blue/green/orange/dark red; Molecular Probes). Single channels are usually shown in inverted grayscale. Annotated movies were converted to QuickTime using ImageJ 1.49s and file sizes adjusted using mpeg-4 compression.

Image and statistical analyses
GraphPad Prism 3.02 (GraphPad Software) was used for statistical analysis and graphical displays of data sets. SigmaPlot 11.0 (Systat Software) was used to plot line scans with data obtained by the MetaMorph line scan plug-in.

To determine the speed of SVs in wt or hookAΔ cells treated with latB, we used time series acquired for middle planes of hyphal tip cells. These time stacks were used to draw 20-µm-long kymographs with segmented lines starting at the apex. Trajectories of moving vesicles were tracked manually on these kymographs. The tracks were used to classify runs into anterograde and retrograde classes and calculate individual speeds using the MetaMorph kymograph plug-in. The mean speeds of anterograde and retrograde runs were significantly different at 28 and 37°C using two-tailed unpaired t testing with Welch’s correction (see the legends to Figures 1 and 2). The speed of EEs in the wt (Figure 6C) was determined with kymographs of mCherry-RabARAB5, similarly to SVs (we observed nearly complete or complete absence of EE runs in hookAΔ, p25Δ, kinaG97E, and uncA116E hyphae).

Apical extension rates (Figure 5D) were calculated from time-lapse series of hyphae growing on the stage in microscopy chambers containing WMM with 0.1% glucose as C source. Images were acquired with the Nomarski optics at 6–12 frames/min over at least 20 min. The resulting time series were used to draw kymographs across the growing tips. In these plots, a growing tip showed up as a diagonal line traced by the extending apex, the slope of which was used to deduce actual apical extension rates (with the MetaMorph kymograph plug-in). Growth rates are exquisitely dependent on the incubation temperature. To optimize growth, we inoculated culture chambers that were incubated overnight at 26°C. These chambers were then transferred to the microscopy room, which had been adjusted to 28–29°C. Upon placement of the culture chamber onto the prewarmed microscope, the objective heater and the stage incubator were set at 29 and 28°C, respectively, and cultures were allowed to grow for a further 60–90 min before acquisition of time series. Under these controlled conditions, the temperature of the medium during image acquisition was 29 ± 0.5°C.

To provide a quantitative estimation of the accumulation of GFP-RabERAB11 SVs in the SPK (Figure 5E), we measured the fluorescence intensity of sum projections of z-stacks (11 planes; z pass, 0.25 µm) in a circular region of interest (24 pixels, −4.5 µm² encompassing the totality of the SPK (subtracting the background determined in an empty equivalent region). Data sets were compared by one-way analysis-of-variance (ANOVA), followed by Bonferroni’s multiple comparison test. Data sets were represented as scatter plots with means and, unless otherwise indicated in the figure legends, 95% confidence interval (CI) bars.

To estimate the flux of SVs circulating on the MTs across the apicalmost 20 µm of wt and mutant cells treated with latB (Figures 3C and 8C), we used kymographs derived from lines that started at the apex and counted manually, using MetaMorph, the number of runs per time unit in each direction in a sample of cells. The resulting data sets, which passed the Kolmogorov–Smirnov normality test, were compared by one-way ANOVA followed by Bonferroni’s multiple comparison test.

The fact that EE runs were less numerous and that the fluorescence was more homogeneous compared with SVs in latB-treated cells facilitated the tracking of the total distance covered by EE runs per unit time in either direction in a sample of five hyphae (Figure 6D; for calculations, 20-µm kymographs were plotted from mCherry-RabARAB5 time series). Data sets were compared by one-way ANOVA followed by Bonferroni’s multiple comparison test.

To obtain an approximate estimation of the flux of SVs attributable to MT transport in untreated wt, kinaG97E, and uncA116E hyphae (Figure 7D), we used GFP-RabERAB11 time series to plot kymographs. Rather than starting at the apex, the 10-µm-long segments that we used for these kymographs excluded the 2- to 4-µm apicalmost region, seeking to analyze flux across slightly subapical regions where AF transport is expected to be less active (although not absent, as some AFs can very likely extend beyond the excluded region). This design also facilitated visualization of the faint runs that we attribute to MT transport, as the strongly fluorescence SPK was excluded. Kymographs were used to count manually the total number of anterograde and retrograde runs. Data sets were analyzed by one-way ANOVA followed by Bonferroni’s multiple comparison test.
Kymographs of NudA-GFP were used to estimate with MetaMorph the MT growth in wt cells (Figure 4C). To acquire time series of NudA-GFP in kinAΔ cells, we used the maximal excitation power of a Leica EL6000 external light source with metal halide lamp for epifluorescence excitation, 400- to 600-ms exposure times, and the maximal gain setting of the ORCA-ER II CCD camera.

ACKNOWLEDGMENTS

We thank Reinhard Fisher and Natalia Requena for the kinA::pyr-4 disruption allele, Berl Oakley for myoEΔ, tubA-GFP, and myoE-GFP alleles, Samara Reck-Peterson for the gene-replaced nudA-3xGFP strain, Elena Reoyo for technical assistance, and four anonymous referees for suggesting valuable improvements to the first version of the manuscript. This work was funded by grants from the Spanish Ministerio de Economía y Competitividad (BIO2012-030965 and BIO2015-65090-R) and the Comunidad de Madrid (Grant S2010/BMD-2144) to M.A.P. and from the National Institutes of Health (RO1 GM097580) and the Uniformed Services University (intramural grant BIO-71-1972) to X.X.

REFERENCES


Molecular Biology of the Cell


Supplemental Materials

Molecular Biology of the Cell

Peñalva et al.
Transport of fungal RAB11 secretory vesicles involves myosin-5, dynein/dynactin/p25 and kinesin-1 and is independent of kinesin-3 and Hook

Supplemental Figure S1
Top: growth phenotypes of the indicated wt and mutant strains at three different temperatures. The different spore colors, which do not cause any effect on vegetative hyphae, are indicated for clarity. Spore colors are used as genetic markers. Bottom: still images illustrating that the wt distribution of SVs labeled with GFP-RabERAB11 is not detectably affected by heat-sensitive mutations in the dynein pathway used in this work. Note the strong accumulation in the SPK and the fainter labeling of TGN cisternae.

Supplemental Figure S2
Dual channel imaging of EEs (mCherry-RabARAB5) and SVs (GFP-RabERAB11) in wild type, hookAΔ and p25Δ cells untreated or treated with latrunculin. The two channels were acquired simultaneously using a Dual Viewer beam splitter and streaming image acquisition to the computer RAM at 400 frames/min. The resulting time-stacks (150 frames, 22.5 sec in total) were used to draw kymographs starting at the tips. Green arrowheads on top of the kymographs indicate the SPK (formed by SVs only without latB treatment) whereas blue arrowheads indicate the apex in those cases in which there is no SPK (mCherry-EE channels and GFP-SVs in latB-treated cells).

Supplemental Figure S3
Steady-state localization of MyoE-GFP in the wild type and in a kinAΔ::pyr-4 strain

Supplemental Figure S4
MTs in kinAΔ::pyr-4 cells contact the cortex in the apical dome, similarly to the wt.

Supplemental Figure S5
Different classes of progeny derived from a cross between myoEΔ and kinAG97E strains. All images are shown at the same magnification. Ascospores from a hybrid cleistothecium were incubated in on microscopy wells before being photographed. Note the wider hyphal diameter of myoEΔ cells compared to the wt.

Supplemental Figure S6
Control experiment demonstrating that latB treatment does not affect the behavior of EEs in the wt or in kinesin mutant strains. Static images on the top and the corresponding linescans correspond to cells treated with latB. These images were obtained from time stacks (100 frames, 400 frames/min) that were used to derive kymographs, starting at the apices. See Figure 6 for EEs in wt and mutant cells that had not been treated with latB.

Supplemental Table S1
Aspergillus nidulans strains constructed for this work
Supplemental Figure S1
Top: growth phenotypes of the indicated wt and mutant strains at three different temperatures. The different spore colors, which do not cause any effect on vegetative hyphae, are indicated for clarity. Spore colors are used as genetic markers. Bottom: still images illustrating that the wt distribution of SVs labeled with GFP-RabE<sup>R641T</sup> is not affected by heat-sensitive mutations in the dynein pathway used in this work. Note the strong accumulation in the SPK and the fainter labeling of TGN cisternae.
Supplemental Figure S2. Dual channel imaging of EEs (mCherry-RabA^{RAB5}) and SVs (GFP-RabE^{GAP}) in wild type, hookAΔ and p25Δ cells untreated or treated with latrunculin. The two channels were acquired simultaneously using a Dual Viewer beam splitter and streaming image acquisition to the computer RAM at 400 frames/min. The resulting time-stacks (150 frames, 22.5 sec in total) were used to draw kymographs starting at the tips. Green arrowheads on top of the kymographs indicate the SPK (formed by SVs only without latB treatment) whereas blue arrowheads indicate the apex in those cases in which there is no SPK (mCherry-EE channels and GFP-SVs in latB-treated cells).
Supplemental Figure S3
Steady-state localization of MyoE-GFP in the wild type and in a \textit{kinA}:\textit{pyr-4} strain
MTs in \( \text{kinA}^{\Delta:}\text{pyr-4} \) cells contact the cortex in the apical dome, similarly to the \text{wt} \.
**Supplemental Figure S5**

Different categories of progeny derived from a cross between *myoEΔ* and *kinA<sup>G97E</sup>* strains. All images are shown at the same magnification. Ascospores from a hybrid cleistothecium were incubated in on microscopy wells before being photographed. Note the wider hyphal diameter of *myoEΔ* cells.
Supplemental Figure S6
Control experiment demonstrating that latB treatment does not affect the behavior of EEs in the wt or in kinesin mutant strains. Static images on the top and the corresponding linescans correspond to cells treated with latB. These images were obtained from time stacks (100 frames, 400 frames/min) that were used to derive kymographs, starting at the apices. See Figure 6 for EEs in wt and mutant cells that had not been treated with latB.
Supplemental Table S1. Strains used in this work

No. (*)  Genotype (**)

4120  rabE::GFP::pyrGAF pyro4A nudA::bar pyrG89
4225  rabE::GFP::pyrGAF yA2 pant0B100 pyrG89 (nkuA::bar?)
4406  paba1 myoE::GFP::pyrGAF (pyrG89?) (nkuA::bar?)
4408  pantoB100 myoE::GFP::pyrGAF (nkuA::argB?) (pyrG89?)
4409  rabE::GFP::rabE::GFP myoE::GFP::pyrGAF (nkuA::argB?) (pyrG89?) (**)
4412  rabE::GFP::rabE::GFP myoE::GFP::pyrGAF nkuA::argB (pyrG89?)
4658  pyrG89 wa::GFP::TubA::GFP::pyro4A nkuA::argB (***)
4788  rabE::GFP::rabE::GFP kinA::pyr-4 (pyrG89?) (nkuA::bar?) (***)
4791  wA::GFP::TubA::GFP::kinA::pyr-4 (pyrG89?) (nkuA::argB?) (yA2?)
4824  wa::GFP::TubA::GFP::myoE::GFP::pyrG89 (nkuA::argB/bar?)
4825  rabE::GFP::rabE::GFP wa::GFP::TubA::GFP::pyrG89 pyro4A (nkuA::argB/bar?) (pyrG89?)
4857  kinA::pyr-4, myoE::GFP::pyrGAF, argB2::argB/alcAp::mCherry::Rabe], pant0B100, hwA1 (yA2?)(nkuA::bar?)(pyrG89?)
4889  kinA::pyrG89 pyro4A nkuA::bar
4891  uncA::pyrG89 pyro4A nkuA::bar
4955  nudA2 pyrG89 wa3 chaA1
4957  nudA5 pyrG89 wa3 chaA1
4958  nudF7 paba1 yA2
4960  nudK17 pyrG89 yA2
4966  rabE::GFP::rabE::GFP chaA1 nudA2 pyrG89
4997  rabE::GFP::rabE::GFP wa3 nudA2 pyrG89 pyro4A
4998  rabE::GFP::rabE::GFP nuda2 pyrG89 pyro4A
4999  rabE::GFP::rabE::GFP nudA5 pyrG89
5000  rabE::GFP::rabE::GFP wa3 nudA5 pyrG89
5001  rabE::GFP::rabE::GFP chaA1 nudA5 pyrG89 pyro4A
5003  rabE::GFP::rabE::GFP chaA1 nudA5 pyrG89 pyro4A
5004  rabE::GFP::rabE::GFP ya2 nudF15 paba1 pyro4A (pyrG89?)
5005  rabE::GFP::rabE::GFP ya2 nudF15 paba1 (pyrG89?)
5006  rabE::GFP::rabE::GFP nudK17 pyro4A (pyrG89?)
5038  rabE::GFP::rabE::GFP kinA::pyrGAF (nkuA::bar?) (pyrG89?)
5039  rabE::GFP::rabE::GFP kinA::pyrGAF ya2 pyro4A pant0B100 (nkuA::bar?) (pyrG89?)
5040  rabE::GFP::rabE::GFP kinA::pyrGAF ya2 pant0B100 (nkuA::bar?) (pyrG89?)
5041  rabE::GFP::rabE::GFP p25::pyrGAF ya2 pyrG89 (nkuA::argB/bar?)
5042  rabE::GFP::rabE::GFP p25::pyrGAF argB2::argB/alcAp::mCherry-RabA ya2 (pyrG89?)(nkuA::argB/bar?)
5045  rabE::GFP::rabE::GFP argB2::argB/alcAp::mCherry-RabA pant0B100 (pyrG89?)(pyrG89?)(nkuA::bar?)
5046  rabE::GFP::rabE::GFP argB2::argB/alcAp::mCherry-RabA (nkuA::argB/bar?) (pyrG89?)
5047  rabE::GFP::rabE::GFP argB2::argB/alcAp::mCherry-RabA pant0B100 pyro4A (nkuA::argB/bar?) (pyrG89?)
5048  rabE::GFP::rabE::GFP hookA::argB2::argB/alcAp::mCherry-RabA pant0B100 (nkuA::argB/bar?) (pyrG89?)
5129  unca(G1116E)::pyrGAF argB2::argB/alcAp::mCherry-RabA nkuA::argB pyrG89 pyro4A wA2
5133  kinA(G917E) HookA::GFP::pyrGAF argB2::argB/alcAp::mCherry-RabA pyrG89 pyro4A wA2 (nkuA::argB pyrG89)
5134  rabE::GFP::rabE::GFP unca(G1116E)::pyrGAF argB2::argB/alcAp::mCherry-RabA (nkuA::argB/bar?) (pyrG89?)
5135  rabE::GFP::rabE::GFP unca(G1116E)::pyrGAF (nkuA::argB/bar?) (pyrG89?)
5187  rabE::GFP::rabE::GFP kinA(G917E) (nkuA::argB/bar?) (pyrG89?)
5188  rabE::GFP::rabE::GFP kinA(G917E) argB2::argB/alcAp::mCherry-RabA wA2 (nkuA::argB/bar?) (pyrG89?)
5189  RabA::pyrGAF unca(G1116E)::pyrGAF argB2::argB/alcAp::mCherry-RabA pyro4A (nkuA::argB/bar?) (pyrG89?)
5210  unca(G1116E)::pyrGAF argB2::argB/alcAp::mCherry-RabA (nkuA::argB/bar?) (pyrG89?)
5270  unca(G1116E)::pyrGAF unca(G1116E)::pyrGAF wa2 pyro4A (nkuA::argB/bar?) (pyrG89?)
5382  rabE::GFP::rabE::GFP unca(G1116E)::pyrGAF pyro4A pant0B100 (nkuA::bar?) (pyrG89?)
5383  rabE::GFP::rabE::GFP unca(G1116E)::pyrGAF ya2 (nkuA::bar?) (pyrG89?)
5388  pykA::pyrGAF pyro4A nbo2 nudA::bar pyrG89
5391  rabE::GFP::rabE::GFP kipA::pyrGAF pyro4A pyrG89 (nkuA::bar?)
5406  myoE::pyrGAF uncaA::pyrGAF pyro4A (pyrG89?) (nkuA::bar?) (pyrG89?)
5464  paba1 uncaA::pyrGAF myoE::GFP::pyrGAF (pyrG89?) (nkuA::bar?)
5465  pyro4A uncaA::pyrGAF myoE::GFP::pyrGAF (pyrG89?) (nkuA::bar?)
5899  kipA::pyrGAF pyro4A nbo2 nudA::bar pyrG89
5931  rabE::GFP::rabE::GFP kipA::pyrGAF pyro4A pyrG89 (nkuA::bar?)
6021  myoE::pyrGAF kipA::pyrGAF nbo2 pyrG89 (nkuA::argB/bar?)
6172  pyro4A nudA::3xFGP::pyrGAF argB2::argB/alcAp::mCherry-RabA pyrG89 (nkuA::bar?)
6173  yA2 kinA::nudA::3xFGP::pyrGAF argB2::argB/alcAp::mCherry-RabA pyrG89 (nkuA::bar?)

(*) Numbers indicate the catalogue designation in the Madrid collection of glycerolized stocks

(***): Question marks indicate that whether the strain carries that particular mutation or not has not been determined. In all cases the presence or absence of these 'non-genotyped' mutations is irrelevant for the conclusions. pyrG89 is complemented by A. fumigatus pyrG transgenes. nkuA has no phenotype by itself other than preventing non-homologous end-joining recombination. In some cases spore color mutations are masked by epistatic relationships.

(***): Notes on source laboratories

4791 The original kinA::pyrE allele used in a parental of this strain was a kind gift from Reinhard Fischer
4409 Its parental myoE::pyrGAF strain was a kind gift from Berl Oakley
4658 A kind strain gift from Berl Oakley
5393 (parental of 6172 and 6173): A kind gift from Samara Reck-Peterson