ABSTRACT Early endosomes are transported bidirectionally by cytoplasmic dynein and kinesin-3, but how the movements are regulated in vivo remains unclear. Here we forward genetic study led to the discovery of VezA, a vezatin-like protein in Aspergillus nidulans, as a factor critical for early endosome distribution. Loss of vezA causes an abnormal accumulation of early endosomes at the hyphal tip, where microtubule plus ends are located. This abnormal accumulation depends on kinesin-3 and is due to a decrease in the frequency but not the speed of dynein-mediated early endosome movement. VezA-GFP signals are enriched at the hypha tip in an actin-dependent manner but are not obviously associated with early endosomes, thus differing from the early endosome association of the cargo adapter HookA (Hook in A. nidulans). On loss of VezA, HookA associates normally with early endosomes, but the interaction between dynein-dynactin and the early-endosome-bound HookA is significantly decreased. However, VezA is not required for linking dynein-dynactin to the cytosolic ΔC-HookA, lacking the cargo-binding C-terminus. These results identify VezA as a novel regulator required for the interaction between dynein and the Hook-bound early endosomes in vivo.

INTRODUCTION The minus end–directed cytoplasmic dynein motor transports organelles/vesicles and proteins/mRNA along microtubules, and its function is important for many cellular processes in both lower and higher eukaryotes (Kardon and Vale, 2009; Perlson et al., 2010; Vallee et al., 2012). However, mechanistically how dynein-mediated transport is regulated in vivo is unclear; in particular, we do not fully understand how dynein interacts with its various cargoes and how its activity is coordinated with that of plus end–directed kinesins (Akhmanova and Hammer, 2010; Fu and Holzbaur, 2014). Several important dynein regulators have been identified, including the dynactin complex, LIS1, and NudE/Nudel (Schroer, 2004; Kardon and Vale, 2009; Valleé et al., 2012), and the functional connection between dynein and LIS1 and that between LIS1 and NudE/Nudel were first suggested by genetic data obtained in the filamentous fungus Aspergillus nidulans (Xiang et al., 1995a; Efimov and Morris, 2000). Although mechanistic studies of these known regulators are critical for our understanding of dynein regulation, identification of novel factors required for dynein-mediated transport in vivo also represents an important complementary approach.

Filamentous fungi are well suited for studying dynein-mediated transport of early endosomes (Granger et al., 2014; Steinberg, 2014; Xiang et al., 2015). As first discovered in Ustilago maydis, the microtubule plus–directed kinesin-3 moves early endosomes toward the plus end, and dynein moves them away from the plus end (Wedlich-Soldner et al., 2002; Lenz et al., 2006; Abenza et al., 2009; Zekert and Fischer, 2009; Egan et al., 2012). In A. nidulans, dynein accumulates at the microtubule plus end near the hyphal tip, and this localization requires kinesin-1 as well as dynactin (Han et al., 2001; Zhang et al., 2003; Egan et al., 2012; Yao et al., 2012). The plus-end accumulation of dynein facilitates transport of early endosomes toward the minus end, most likely by enhancing the chance of dynein–early endosome interaction (Lenz et al., 2006; Zhang et al., 2010; Schuster et al., 2011a,b) and/or possibly by...
allowing the interaction to occur at the hyphal tip region where a positive regulator may be located. The association between dynein and early endosomes requires the dynactin complex, particularly the β-helix–containing p25 subunit (Eckley et al., 1999; Zhang et al., 2011; Yeh et al., 2013), which is not required for nuclear distribution (as first shown in Neurospora crassa) but is required for early endosome transport in both A. nidulans and mammalian cells (Lee et al., 2001; Zhang et al., 2011; Yeh et al., 2012). Recently genetic screens in A. nidulans and U. maydis led to the discovery of the Hook-FTS-FHIP complex, a complex first found in higher eukaryotes (Kramer and Phistry, 1996; Xu et al., 2008), as critical for linking dynein-dynactin to early endosomes, and U. maydis Hook (Hok1) also plays an important role in recruiting kinesin-3 to these cargoes (Bielska et al., 2014; Yao et al., 2014; Zhang et al., 2014). However, it has not been clear whether additional factors are required for regulating the bidirectional transport of early endosomes.

Here we report the identification of a novel factor, vezatin-like protein in A. nidulans (VezA), which is critical for dynein-mediated early endosome transport. VezA contains a vezatin domain, as well as two predicted transmembrane domains similar to those in the mammalian vezatin protein. Vezatin is involved in neuronal functions, stabilization of cell–cell adhesion, and Listeria entry into cells, and it is also a putative tumor suppressor and linked with endometriosis (Kussel-Andermann et al., 2000; Sousa et al., 2004; Bahloul et al., 2009; Sanda et al., 2010; Danogl et al., 2012; Miao et al., 2013; Pagliardini et al., 2015). Vezatin interacts with FERM domain–containing proteins (Kussel-Andermann et al., 2000; Bahloul et al., 2009), and it also interacts specifically with the GTP-bound ADP-ribosylation factor 6, Arf6 (Sanda et al., 2010). Although the mammalian vezatin proteins have never been implicated in microtubule-based transport, loss of VezA in A. nidulans causes an obvious defect in dynein-mediated transport of early endosomes. Of interest, VezA is enriched at the hyphal tip but does not appear to colocalize with early endosomes, which is in contrast to HookA (Hook in A. nidulans), which is obviously associated with early endosomes. Of importance, although VezA does not affect HookA–early endosome association, it affects the interaction between dynein and HookA when HookA is attached to the early endosome. Thus VezA is a novel factor regulating dynein–early endosome interaction in vivo.

RESULTS

Identifying VezA as a novel factor required for early endosome distribution

We ultraviolet (UV)-mutagenized A. nidulans conidiospores and screened for early endosome distribution (eed) mutants, which exhibit an abnormal accumulation of early endosomes at the hyphal tip. Because mutations that affect the overall function of dynein severely inhibit colony growth but mutants that are more specifically defective in early endosome movement form relatively healthy colonies (Zhang et al., 2011, 2014), we selected the mutants that formed relatively healthy colonies. Moreover, to limit the chance of finding genes that affect the general function or localization of dynein, we collected only those mutants in which dynein localization at the microtubule plus ends can be seen and dynein-mediated nuclear distribution appears normal. Previous screens allowed us to identify the eedA and eedB genes, which encode HookA and HipA, respectively (Yao et al., 2014; Zhang et al., 2014). In the present study, we selected a mutant, eed27, whose colonies appear slightly more compact than that of the wild type (Figure 1A), but whose colony phenotype is even subtler than that of the ∆hipA or ∆hookA mutant. Microscopic observations of the eed27 mutant revealed an abnormal accumulation of early endosomes at a vast majority of hyphal tips (>80%; n > 100; Figure 1, B and C, and Supplemental Movies S1 and S2), whereas plus-end comets of dynein are present (Figure 1B). Despite the abnormal accumulation at the hyphal tip in the mutant, mCherry-RabA (Rab5 homologue)–labeled early endosomes can be seen moving in both directions (Figure 1C), which differs from the ∆hookA mutant, in which we almost never observed movement. To determine whether the newly identified eed27 mutation is in the previously identified genes involved in dynein-mediated early endosome transport but not in the overall function of dynein, for example, p25 of dynactin, hookA, or hipA, we crossed the eed27 mutant with the ∆p25, ∆hookA, or ∆hipA mutant. All of these crosses generated wild-type progeny, indicating that the eed27 mutation is not in any of these known genes, and thus we named the mutation eedC27.

To identify the eedC27 mutation, we used a combination of classical genetic and whole-genome sequencing approaches. Whole-genome sequencing was done using the genome sequencing and bioinformatic service of Otogenetics (www.otogenetics.com). We also used the software Tablet (version 1.13.12.17, James Hutton Institute; ics.hutton.ac.uk/tablet/download-tablet/tablet-release-notes/) for visualizing sequence assemblies and alignments (Milne et al., 2013). To facilitate gene identification, we used the parasexual cycle of A. nidulans to map the eedC27 mutation to chromosome VIII, and this combination of genetic and genome-sequencing approaches allowed us to identify the gene without any ambiguity. The eedC gene corresponds to An10076, which encodes a novel protein with 615 amino acids. Of interest, the protein contains a vezatin domain (amino acids [aa] 150–380; Figure 1D) and shows weak similarity to the mammalian vezatin, which was initially identified as a protein binding to the FERM domain of myosin VIIA to bring it close to the cadherin–catenin complex (Kussel-Andermann et al., 2000; Supplemental Figure S1). Two transmembrane domains, aa 142–164 and 177–195, were predicted by the SMART program (smart.embl-heidelberg.de; Figure 1D and Supplemental Figure S1). Note that the mammalian vezatin also contains two transmembrane domains (aa 134–158 and 166–188; Bahloul et al., 2009). The eedC27 mutation is a deletion of a nucleotide T, creating a frameshift after aa 276. Because of the sequence similarity to vezatin, we named the eedC gene vezA and the encoded protein VezA.

To confirm that VezA is required for early endosome distribution, we made the deletion mutant ∆vezA (Supplemental Figure S2), and performed a detailed analysis on its phenotype. The ∆vezA mutant showed a colony phenotype nearly identical to that of the original UV-generated eedC27 mutant (Figure 2A). Just like the original mutant, the ∆vezA mutant also exhibited an abnormal accumulation of mCherry-RabA–labeled early endosomes at the hyphal tip (Figure 2B). Dynein comets, which represent the microtubule plus-end accumulation of dynein, were present (Figure 2B). In addition, ∆vezA exhibited a normal pattern of nuclear distribution (Figure 2C). Taken together, these results indicate that VezA is not essential for the overall function and localization of dynein but is critical for dynein-mediated early endosome transport.

To determine whether VezA functions solely in a genetic pathway with HookA or p25, we constructed ∆vezA ∆hookA and ∆vezA ∆p25 double mutants. Of interest, the ∆vezA ∆hookA and ∆vezA ∆p25 double-mutant colonies looked slightly more compact than the ∆hookA or ∆p25 single mutants, respectively (Supplemental Figure S2C), suggesting that VezA might participate in additional pathways beyond that of the p25/HookA–controlled early endosome transport.

Endocytosis in fungi involves actin patches (Engqvist-Goldstein and Drubin, 2003; Gachet and Hymans, 2005; Michelot et al., 2010;
However, endocytosis along the whole hyphae, as evidenced by uptake of the dye FM4-64, has been observed to occur in live cells (Peñalva, 2005). The newly formed endocytic vesicles would fuse with Rab5 (RabA and RabB of A. nidulans)–positive early endosomes, and, as first shown in U. maydis (Wedlich-Soldner et al., 2002; Lenz et al., 2006), they would be transported toward the microtubule plus end by kinesin-3 before being moved back by dynein (Abenza et al., 2009, 2010; Zekert and Fischer, 2009; Egan et al., 2012).

Unlike ∆hookA, in which motile early endosomes were almost never observed, we were able to detect some motile early endosomes in the ∆vezA mutant (Figure 2D). We did a quantitative analysis of early endosome movement within a region ∼5 μm from the hyphal tip where microtubule plus ends all face the hyphal tip (Xiang et al., 2015). We found that the frequency of dynein-mediated movements (away from the hyphal tip) significantly decreased upon loss of VezA. The frequency of movements to the hyphal tip (kinesin-3 mediated) also decreased, which is similar to the situation in the ∆p25 mutant (Figure 2E; Zhang et al., 2011). To determine whether the hyphal-tip accumulation of early endosomes in the ∆vezA mutant is due to a defect in dynein motor activity, we also measured the speed of dynein-mediated early endosome movement. We found that the speed is clearly not reduced (Figure 2F), indicating that VezA does not affect dynein motor activity. To determine directly whether the abnormal hyphal-tip accumulation of early endosomes in the ∆vezA mutant depends on kinesin-3, we introduced the ∆uncA (kinesin-3) allele (Zekert and Fischer 2009) into the strain containing the ∆vezA allele. In the ∆vezA ∆uncA double mutant, the hyphal-tip accumulation of early endosomes in the ∆vezA single mutant was no longer observed (Figure 3A), and our quantitation of the hyphal-tip mCherry-RabA signals in the ∆vezA ∆uncA double mutant is consistent with a much reduced accumulation of early endosomes at the hyphal tip compared with that in the ∆vezA single mutant (Figure 3, A and B). These results indicate that VezA is involved in dynein-mediated early endosome transport after early endosomes are moved toward the hyphal tip by kinesin-3.

VezA proteins are enriched at the hyphal tip in an actin-dependent manner
To localize VezA, we constructed a strain in which the endogenous vezA gene is replaced by the vezA–green fluorescent protein (GFP) fusion gene (Supplemental Figure S3A). For comparison, we also
constructed a strain in which the endogenous vezA gene is replaced by the ΔTM-vezA-GFP fusion gene missing the two predicted transmembrane domains (Supplemental Figure S3B). The VezA-GFP protein is functional, as cells expressing it exhibited a normal pattern of early endosome distribution (Figure 4A and Supplemental Movies S3 and S4). In contrast, the ΔTM-vezA-GFP strain exhibited a clear defect in early endosome distribution, as similarly exhibited by the ΔvezA mutant (Figure 4A and Supplemental Movie S5). VezA-GFP signals were significantly enriched at the hyphal tip in many cells (Figure 4B; Supplemental Movies S6–S8 show different examples). To visualize the plasma membrane, we stained the cells with FM4-64, a lipophilic dye that reveals the fungal plasma membrane if the cells are treated briefly (Galindo et al., 2012; Kilaru et al., 2015). However, the FM4-64 treatment seemed to disturb the hyphal-tip GFP signals, and thus we took the images of VezA-GFP before adding the dye and then took the FM4-64 images. In some of these cells, we saw a bright, dot-like GFP signal very close to the hyphal apex (Figure 4B). This dot-like signal seemed relatively stable (as shown in the kymograph in Figure 4B and in Supplemental Movie S6), but sometimes it was seen to move around (Supplemental Movie S7). Slightly behind it were VezA-GFP signals that were more dynamic but seemed to only undergo short-distance movements.
We next determined whether the hyphal-tip enrichment of VezA depends on microtubules or the actin cytoskeleton by using benomyl, a microtubule-depolymerizing drug, or latrunculin B, an actin polymerization inhibitor, respectively. Microtubules are not essential for polarized growth but are required for rapid hyphal elongation (Horio and Oakley, 2005), and, thus, short hyphae were seen after overnight treatment with benomyl at a concentration (1.2 μg/ml) that depolymerized all microtubules (unpublished data). Although the shapes of the hyphal tips looked less focused in benomyl-treated cells than in untreated cells, the hyphal-tip enrichment of VezA-GFP signals was still present (Figure 4G). In contrast, because actin polymerization is essential for polarized growth, we treated the VezA-GFP-containing cells with latrunculin B for only 15 min. Of interest, the VezA-GFP signals were quickly dispersed and the hyphal-tip enrichment was no longer observed after the treatment (Figure 4H), suggesting that the hyphal-tip localization of VezA is supported by the actin cytoskeleton. We would add a note of caution, however; because the hyphal-tip VezA-GFP signals also seemed to be perturbed by FM4-64 treatment, it is possible that they represent VezA localization to membranous structures that are partially tethered by the actin cytoskeleton.

**VezA does not affect HookA–early endosome association but affects the interaction between early endosome–bound HookA and dynein-dynactin.**

As recently discovered in *A. nidulans* and *U. maydis*, Hook proteins link dynein to early endosomes, and in *A. nidulans*, HookA interacts with early endosomes via FhipA and FtsA (Bielska et al., 2014; Yao et al., 2014; Zhang et al., 2014). To determine whether VezA affects HookA–early endosome association, we introduced the HookA-GFP fusion into the ∆vezA mutant by genetic crossing. We found that HookA-GFP proteins largely colocalize with mCherry-RabA–labeled early endosomes accumulated at the hyphal tip and individual early endosomes along the hyphap (Figure 5A). To compare quantitatively the early endosome–associated HookA-GFP signals in the ∆vezA mutant and wild type, we treated wild-type and ∆vezA cells with benomyl overnight, which allowed us to measure the ratio of HookA-GFP to mCherry-RabA on early endosomes that are not clustered together at the hyphal tip. Our analysis showed that the values from the wild type and the mutant were nearly identical, indicating that VezA does not affect the association between HookA and early endosomes (Figure 5, B and C).

We next determined whether VezA affects the ability of HookA to interact with dynein, using biochemical pull-down assays. For this experiment, we first introduced the ∆C-HookA-GFP fusion into the ∆vezA mutant by genetic crossing. As shown previously, HookA–dynein-dynactin interaction does not need the C-terminal early endosome–binding site of HookA, and the cytosolic ∆C-HookA-GFP fusion pulls down dynein-dynactin effectively (Zhang et al., 2014). Thus the pull-down experiments using the cytosolic ∆C-HookA-GFP allow us to determine whether VezA mediates the interaction between HookA and dynein-dynactin. Our Western analyses indicate that ∆C-HookA-GFP is able to pull down normal amounts of dynein, dynactin, and the dynein-binding protein NudF/LIS1 in the ∆vezA extract (Figure 5D). Thus VezA is not required for linking dynein-dynactin to the cytosolic ∆C-HookA. We next examined whether early endosome–bound, full-length HookA can pull down dynein and dynactin. For this experiment, we used strains containing HookA-GFP and HookA-GFP/∆vezA, respectively. Because HookA-GFP is physically associated with early endosomes in the absence of VezA, it can be used to pull down early endosomes in the absence of detergent and allow us to determine whether dynein...
defective. This result is consistent with the decrease in the frequency
of dynein-mediated transport in the ΔvezA mutant (Figure 2E).
Together the results obtained with ΔC-HookA and full-length HookA
indicate that whereas VezA is required for regulating the interaction
and dynactin are able to associate with HookA-bound early endosomes. Of interest, in this experiment, the amounts of pulled-down
dynein, dynactin, and NudF/LIS1 are significantly reduced (Figure 5,
E and F), indicating that dynein–early endosome interaction is

FIGURE 4: The functional VezA-GFP fusion proteins are concentrated at the hyphal tip. (A) The normal distribution of mCherry-RabA–labeled early endosomes in the vezA-GFP strain indicates that VezA-GFP is functional. In contrast, the ΔTM-vezA-GFP strain shows an abnormal accumulation of early endosomes at the hyphal tip, indicating that the predicted transmembrane domains are important for VezA function. Kymographs are shown below to indicate that early endosomes move normally in the wild-type and vezA-GFP strains but largely accumulate at the hyphal tip in the ΔTM-vezA-GFP strain. The images and kymographs are from Supplemental Movie S3 (mCherry-RabA in the wild-type control), Supplemental Movie S4 (mCherry-RabA in the vezA-GFP strain), and Supplemental Movie S5 (mCherry-RabA in the ΔTM-vezA-GFP strain). (B) Images of VezA-GFP and FM4-64 in the same hypha. Glucose-containing medium was used, which prevented the expression of the alcA-controlled mCherry-RabA. One kymograph is shown below to illustrate the hyphal-tip enrichment of the VezA-GFP signals. The merged image (green, VezA-GFP; red, FM4-64) and the kymograph below are from Supplemental Movie S6. Supplemental Movies S7 and S8 are provided to show two other examples of VezA-GFP accumulation at the hyphal tip. (C) Images of a ΔTM-VezA-GFP–containing hypha. Both GFP and the mCherry-RabA signals are shown for the same hypha. Right, Western blot showing that the ΔTM-VezA-GFP fusion proteins are stably expressed. (D) Images of GFP-VezA and mCherry-RabA in the same hypha. (E) Images of VezA-GFP–containing hypha cultured overnight in medium containing 1.2 μg/ml benomyl. (G) Images of a VezA-GFP–containing hypha treated with 100 μM latrunculin B (in DMSO) for 15 min and a control hypha treated in the same way but with DMSO only. Yellow dotted lines show the hyphal shape. Bars, 5 μm.
FIGURE 5: VezA does not affect HookA–early endosome association but affects the physical interaction between the early endosome–bound HookA and dynein-dynactin. (A) Localization of HookA-GFP in the ΔvezA mutant. HookA-GFP largely colocalizes with mCherry-RabA–labeled early endosomes at the hyphal tip in the ΔvezA mutant. Arrows indicate the hyphal tip area where both the GFP and mCherry signals accumulate and largely overlap. Arrowheads indicate the individual early endosomes with HookA-GFP. Bars, 5 μm. (B) HookA-GFP colocalizes with mCherry-RabA–labeled early endosomes in benomyl-treated wild-type and ΔvezA cells. Yellow dotted lines show the hyphal shape. Bars, 5 μm. (C) A quantitative analysis of the intensity ratios of HookA-GFP to mCherry-RabA in benomyl-treated wild-type and ΔvezA cells. The mean ± SD values are 0.0460 ± 0.0232 for wild type (n = 20) and 0.0456 ± 0.0339 for ΔvezA (n = 20), and the mean value of the mutant is not different from that of the wild type at p = 0.05. (D) Western analysis showing that VezA does
between early endosome–bound HookA and dynein-dynactin in vivo, it is unlikely to be required for physically bridging the interaction. Thus VezA is more likely a regulator than an additional cargo adapter.

**DISCUSSION**

The identification of VezA, a protein that localizes at the hyphal tip, for early endosome distribution clearly adds important conceptual complexity to our understanding of dynein-based transport: it suggests that dynein–cargo interaction in vivo requires novel factors beyond cargo adapters. However, although the localization of VezA at the hyphal tip region where microtubule plus ends are located is consistent with VezA being a positive regulator of the dynein–early endosome interaction, exactly how VezA regulates dynein–early endosome interaction needs to be studied further. The fungal hyphal-tip area is crowded with the actin cytoskeleton and many vesicles (Harris et al., 2005; Steinberg, 2007; Fischer et al., 2008; Schuster et al., 2012; Pantazopoulou et al., 2014; Riquelme and Sanchez-Leon, 2014; Zhu and Lee, 2014; Peñalva, 2015; Schlusthaus et al., 2015), and VezA molecules are enriched at the hyphal-tip area in an actin-dependent manner. However, the latrunculin treatment that dispersed VezA within 15 min did not appear to affect significantly the frequency of dynein-mediated movement within 1 h (1.94 ± 0.998/16 s in latrunculin [n = 31] vs. 2.06 ± 1.03/16 s in dimethyl sulfoxide [DMSO]; n = 35). This result suggests that VezA may remain functional after it dissociates from the actin cytoskeleton at the hyphal tip. Alternatively, the cellular effect of VezA may be relatively long lasting, or VezA may only function in the context of the normal actin cytoskeleton at the hyphal tip. Although some vesicles fully capable of binding dynein are more likely to be transported to the actin cytoskeleton after kinesin-mediated transport (Pantazopoulou et al., 2014), as first shown in melanosocytes (Wu et al., 1998), early endosomes appear to be transported back directly by dynein. However, the directional switch from kinesin-3–mediated to dynein-mediated transport is not instant and may require complicated regulation (Bielska et al., 2014). In U. maydis, before an early endosome binds dynein, half of the bound kinesin-3 molecules detach from it, whereas the number of bound Hook molecules remains constant (Bielska et al., 2014). Whether this is functionally relevant remains to be determined, but given that fungal kinesin-3s contain a PH domain and other PH domain–containing kinesin-3 interact with phosphatidylinositol 4,5-bisphosphate (PIP2; Klopfenstein et al., 2002; Klopfenstein and Vale, 2004), it is possible that the early endosome lipid composition may affect kinesin-3 binding (Bielska et al., 2014), thereby affecting the direction switching process. Of interest, mammalian vezatin interacts specifically with the GTP-bound form (the active form) of kinesin-1 (Montagnac et al., 2009). Future work is needed to address the possible interaction between VezA and the Arf6 homologue in A. nidulans (Lee et al., 2008; Lee and Shaw, 2008).

The functional connection between vezatin and cytoplasmic dynein in higher eukaryotes has not been established. Vezatin in mammalian cells is considered to be a transmembrane protein, but its localization at the cell–cell contact sites is supported by the actin cytoskeleton (Kussel-Andermann et al., 2000; Bahloul et al., 2009), similar to the actin-dependent localization of VezA. Of interest, cytoplasmic dynein has also been found to localize to the cortex of the adherens junctions in an actin-dependent manner, although the function of dynein in there has been implicated in tethering microtubules (Ligon et al., 2001; Ligon and Holzbaur, 2007). Vezatin is expressed in neuronal cells and has been implicated in regulating dendritic morphology in hippocampal neurons (Sanda et al., 2010; Danglot et al., 2012). Cytoplasmic dynein is also important for dendritic morphogenesis in Drosophila dendritic arborization neurons via its function in transporting Rab5-positive early endosomes (Satoh et al., 2008). However, vezatin has never been shown to be important for the movement of Rab5-positive early endosomes in mammalian cells. Based on the present study, it will be worthwhile to test whether vezatin is involved in dynein-mediated intracellular transport of membranous cargoes in higher eukaryotes.

**MATERIALS AND METHODS**

**A. nidulans strains, media, and mutagenesis**

*A. nidulans* strains used in this study are listed in Table 1. For biochemical experiments, yeast extract plus glucose plus uridine and uracil liquid medium or minimal plus fructose liquid medium plus supplements was used. UV mutagenesis on spores of *A. nidulans* strains was done as previously described (Willins et al., 1995; Xiang et al., 1999). For 4′,6-diamidino-2-phenylindole (DAPI) staining of nuclei, cells were incubated in liquid minimal medium containing 1% glycerol plus supplements overnight at 32°C. For live-cell imaging experiments, liquid minimal medium containing 1% glycerol plus supplements was used, and cells were cultured at 32°C overnight and observed at room temperature. For some experiments on VezA-GFP, liquid minimal medium containing 0.1% (wt/vol) glucose was used to incubate cells overnight at room temperature; under this condition, alcA-controlled mCherry-RabA is not expressed.

**REFERENCES**

(Bielska et al., 2014)
Genotype

This work

ΔgpdA

This work

Δ

This work

ḎhookA-AfpyrG; argB2::[argB*-alcAp::mCherry-RabA]; ΔnuKuA::argB; pyrG89; pyrA4; wA2

This work

ZX223

Δp25:AfpyrG; GFP-nudAIC; argB2::[argB*-alcAp::mCherry-RabA]

This work

XY105

eedC27; GFP-nudAIC; argB2::[argB*-alcAp::mCherry-RabA]; ΔnuKuA::argB; pyrG89; pyrA4; yA2

This work

XY136

ΔvezA::AfpyrG; GFP-nudAIC; argB2::[argB*-alcAp::mCherry-RabA]; ΔnuKuA::argB; pyrG89; pyrA4; yA2

This work

XY139

ΔnuC::pyrA4; GFP-nudAIC; argB2::[argB*-alcAp::mCherry-RabA]; pyrG89; ΔnuKuA::argB?

This work

XY144

ΔvezA::AfpyrG; HookA-GFP-AfpyrG; argB2::[argB*-alcAp::mCherry-RabA]; pabaA1; pyrA4; pyrG89? ΔnuKuA::argB?

This work

XY140

ΔvezA::AfpyrG; ΔnuC::pyrA4; GFP-nudAIC; argB2::[argB*-alcAp::mCherry-RabA]; yA2; ΔnuKuA::argB?

This work

XY161

ΔvezA::AfpyrG; ΔhookA::AfpyrG; GFP-nudAIC; argB2::[argB*-alcAp::mCherry-RabA]; yA2; ΔnuKuA::argB?

This work

XY163

vezA-GFP-AfpyrG; argB2::[argB*-alcAp::mCherry-RabA]; ΔnuKuA::argB; pyrG89; pyrA4; wA2

This work

XY165

vezA-GFP-AfpyrG; ΔhookA::AfpyrG; argB2::[argB*-alcAp::mCherry-RabA]; ΔnuKuA::argB?: pyrG89?

This work

XY166

ΔvezA::AfpyrG; ΔC-hookA-GFP-AfpyrG; argB2::[argB*-alcAp::mCherry-RabA]; ΔnuKuA::argB?

This work

XY167

ΔTM-vezA-GFP-AfpyrG; argB2::[argB*-alcAp::mCherry-RabA]; ΔnuKuA::argB; pyrG89; pyrA4; wA2

This work

XY170

ΔvezA::AfpyrG; Δp25:AfpyrG; GFP-nudAIC; argB2::[argB*-alcAp::mCherry-RabA]; ΔnuKuA::argB?: pyrG89? yA2

This work

XY171

gpdA<sup>Δmnc</sup>:GFP-vezA; argB2::[argB*-alcAp::mCherry-RabA]; ΔnuKuA::argB; pyrA4; wA2

This work

Source

Qiu et al. (2013)

Qiu et al. (2013)

Zhang et al. (2014)

Zhang et al. (2014)

Zekert and Fischer (2009)

Zhang et al. (2011)

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Markers not confirmed are indicated by question marks.

**TABLE 1:** *A. nidulans* strains used in this study.

**Live-cell imaging and analyses and drug treatments**

Fluorescence microscopy of live *A. nidulans* hyphae was as described (Yao et al., 2014; Zhang et al., 2014). All images were captured at room temperature using an Olympus (Center Valley, PA) IX70 inverted fluorescence microscope linked to a PCO/ Cooke Corporation (Romulus, MI) Sensicam QE cooled charge-coupled device camera. An UPlanApo 100× objective lens (oil) with a 1.35 numerical aperture was used. A filter wheel system with GFP/mCherry-ET Sutter series with high transmission (Biovision Technologies, Exton, PA) was used. IPLab software was used for image acquisition and analysis.

For most imaging experiments, the LabTek Chambered #1.0 borosilicate coverglass system from Nalge Nunc International (Rochester, NY) was used. For experiments with FM4-64 staining, we used Delta T dishes from Biopetchs (Butler, PA), which can be fixed on the Biopetchs heating stage so that the dish does not move during the addition of the dye. About 2 μl of FM4-64 (200 μM) was added to 1.2 ml of medium, and the cells were stained for ~1–5 min at room temperature. Because addition of the dye slightly disrupted the VezA-GFP signals, we took images of VezA-GFP first before adding the dye. Benomyl was used at the final concentration of 1.2 μg/ml, and latrunculin B (Sigma-Aldrich, St. Louis, MO) was used at the final concentration of 100 μM. For the short-time latrunculin B treatment, we followed a similar procedure to that described in Taheri-Talesh et al. (2008). Specifically, half of the culture medium was removed from the observation chamber to be mixed well with latrunculin and added back to the observation chamber. The solvent for latrunculin B, DMSO, was used as a control.

To quantitate the mCherry-RabA signals at hyphal tip, we chose a region of interest (ROI) covering an area from the hyphal apex to ~2 μm behind it and obtained the mean intensity value using IPLab software. To quantitate the signal intensity of HookA-GFP in relation to that of mCherry-RabA in benomyl-treated cells, we chose an ROI covering just the early endosome of interest and obtained the sum values of both GFP and mCherry signals using IPLab software. For each measurement, the exact same ROI box was dragged outside of the cell to take the background value, which was then subtracted from the sum value.

**Construction of the ΔvezA mutant**

For constructing the ΔvezA mutant, the following oligos were used to make the ΔvezA construct with the selective marker pyrG from *Aspergillus fumigatus*, AfpyrG, in the middle of the linear construct (Szewczyk et al., 2006): UUTRf (5′-AAACATCTTTTGGTCTCATCAATT-3′), UUTRr (5′-CTCTGAAACCCGGATATTCAAT-3′), FUSf (5′-CTCTGAAACCCGGATATTCAAT-3′), FUSr (5′-CGTGAAGATCAAGGCTTGTCTCTTCTTG-3′), and UTR (5′-ACAAGGCTTGTCTCTTCTTG-3′). We then used two oligos, UUTRf (5′-TGGCAGATTGGCTTTAGTCTC-3′) and UTRr (5′-AACGACATCAGGAGAGTCG-3′), for a fusion PCR to generate the ΔvezA-AfpyrG fragment that we used to transform into the wild-type strain RQ2. The strain containing the ΔvezA allele was confirmed by PCR of genomic DNA, and the primers used for verifying the correct integration were UUTRf and AfpyrG3 (5′-GTTGCCAGGATGGAGGTATT-3′), and AfpyrG5 (5′-AGCAATAGGACTGATC-3′) and UTR (Supplemental Figure S2).

**Construction of the strain containing VezA-GFP**

For constructing the VezA-GFP fusion, we first used the following oligos to amplify vezA genomic DNA and the GFP-AfpyrG fragment that we used to transform into the wild-type strain RQ2.

The strain containing the ΔvezA allele was confirmed by PCR of genomic DNA, and the primers used for verifying the correct integration were UUTRf and AfpyrG3 (5′-GTTGCCAGGATGGAGGTATT-3′), and AfpyrG5 (5′-AGCAATAGGACTGATC-3′) and UTR (Supplemental Figure S2).
fusión from the plasmid pFNO3 (deposited in the Fungal Genetics Stock Center, [sbs.umkc.edu/research_fungal_stock_center.cfm]) by Stephen Osmani [Department of Molecular Genetics, The Ohio State University, Columbus, OH]; McCluskey et al., 2010; Yang et al., 2004): vgORF (5′-TCATGCTGCTGCTGCTGTA-3′), vgORFr (5′-GAGGCTGTGAGCCTCTGTTGTA-3′); vgFUSf (5′-ATTCAAAACCGCTACAAGCCTCGAGGCAGCGCTGAG-3′), vgFUSr (5′-TCAGAGCTGTAGCCGTCTTGCTGAGGAAGGCGACTGATGTA-3′); vgUTRf (5′-TGATCCCTACACACAGTCGA-3′), and UTRr (5′-ATGCTCCTGCTCAATGTTCCA-3′). We made an oligos, vgORFf (5′-CATGACAGAGCCAGGAGTGAG-3′) and UTRri (5′-AACGACATCCAGGAGTGCTGTA-3′), for a fusion PCR to generate the VezA-GFP-AfpyrG fragment that we used to transform into a wild-type strain RQ54. The strain containing the VezA-GFP fusion gene integrated correctly at the vezA locus was confirmed by PCR of genomic DNA using primers vgORFf and UTRr.

### Construction of the strain containing ΔTM-VezA-GFP

We made a ΔTM-vezA-GFP strain containing the ΔTM-vezA-GFP allele in the vezA locus, and in this strain, the two predicted transmembrane domains of VezA (Supplemental Figure S1) and the short stretch of amino acids in between are deleted and GFP is linked at the C-terminus of the mutant protein. The following oligos were used to make two fragments with the vezA-GFP genomic DNA as template: UUTRf (5′-AACATCTTTCTCTCATCAATCAA-3′), dTM-fuser (5′-CGGTTAAGTATTGCGAGGATG-3′), dTMfusef (5′-CTGACCTTCCTGCATATCACAACCGAGCCAGTGCGTC-3′), and UTRr (5′-ATGCGCTCCGTCAGATGAGCA-3′).

### Construction of the strain containing gpdAΔmin::GFP-VezA

We made a strain containing the gpdAΔmin::GFP-vezA allele in the vezA locus. In the gpdAΔmin::GFP-vezA strain, GFP is fused to the N-terminus of VezA, and expression of the fusion gene is driven by the gpdAΔmin promoter (Pantazopoulos and Peñalva, 2009). By using the DNA synthesis service of GenScript USA (Piscataway, NJ), we made an -1.3 kb DNA fragment containing the 1-kb region before the start codon of VezA, followed by the 355-base pair gpdAΔmin promoter, the 0.7-kb GFP gene, and the 1-kb region after the start codon of VezA. This -3.1 kb fragment was amplified using primers gpdGVi (5′-CAATTCAAGTGCTCCTGGATAC-3′) and gpDGvi (5′-TGCTGTAATCTGTTCTGTA-3′) and cotransformed into RQ54 with a 3-kb fragment containing the A. nidulans pyrG gene, which was amplified from wild-type genomic DNA using primers pyrGF (5′-CCTGATACCCGAGATGTA-3′) and pyrGR (5′-GGGCGAGGGTCAGTAGAAAACA-3′). The strain containing the gpdAΔmin::GFP-vezA fusion that integrated correctly at the vezA locus was confirmed by PCR of genomic DNA using primers gpdGVi (5′-TGCTGTAATCTGTTCTGTA-3′) and gpDGvi (5′-ATTCCGGATACCCGAGATGTA-3′).

### Biochemical pull-down assays to examine the interactions between HookA and dynein-dynactin

The pMACS GFP-tagged protein isolation kit (Milenyi Biotec, San Diego, CA) was used to determine whether GFP-tagged HookA or ΔC-HookA pulls down dynein and dynactin. This was done as described previously (Zhang et al., 2014). Strains were grown overnight in yeast extract/glucose or minimal medium containing 0.4% fructose (wt/vol). About 0.4 g of hyphal mass was harvested from overnight culture for each sample, and cell extracts were prepared using a lysis buffer containing 50 mM Tris- HCl, pH 8.0, and 10 μg/ml a protease inhibitor cocktail (Sigma-Aldrich). Cell extracts were centrifuged at 8000 × g for 15 min and then 16,000 × g for 15 min at 4°C, and supernatant was used for the pull-down experiment. To pull down GFP-tagged protein, 25 μl of anti-GFP MicroBeads was added to the cell extracts for each sample and incubated at 4°C for 30 min. The MicroBeads/cell extract mixture was then applied to the μColumn, followed by gentle wash with the lysis buffer as used for protein extraction (Milenyi Biotec). Preheated (95°C) SDS– PAGE sample buffer was used as elution buffer. Western analyses were performed using the alkaline phosphatase (AP) system, and blots were developed using AP color development reagents from BioRad (Hercules, CA). Quantitation of the protein band intensity was done using IPLab software as described previously (Yao et al., 2012; Qiu et al., 2013). The intensity ratios of the pulled-down dynein HC, dynactin p150, or NudF/LIS1 to GFP-labeled HookA or ΔC-HookA proteins were calculated. The antibody against GFP was from Clontech (Mountain View, CA; polyclonal). The antibodies against dynein HC, dynactin p150, and NudF/LIS1 were described previously (Xiang et al., 1995a,b; Zhang et al., 2008).

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### REFERENCES


