The p25 subunit of the dynactin complex is required for dynein–early endosome interaction

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Cyttoplasmic dynein transports various cellular cargoes including early endosomes, but how dynein is linked to early endosomes is unclear. We find that the Aspergillus nidulans orthologue of the p25 subunit of dynactin is critical for dynein-mediated early endosome movement but not for dynein-mediated nuclear distribution. In the absence of NUDF/LIS1, p25 deletion abolished the localization of dynein–dynactin to the hyphal tip where early endosomes abnormally accumulate but did not prevent dynein–dynactin localization to microtubule plus ends. Within the dynactin complex, p25 locates at the pointed end of the Arp1 filament with Arp11 and p62, and our data suggest that Arp11 but not p62 is important for p25–dynactin association. Loss of either Arp1 or p25 significantly weakened the physical interaction between dynein and early endosomes, although loss of p25 did not apparently affect the integrity of the Arp1 filament. These results indicate that p25, in conjunction with the rest of the dynactin complex, is important for dynein–early endosome interaction.

Introduction

Intracellular membrane trafficking is essential for cell function, and how motor proteins are targeted to various membranous cargoes to power their movement is a question of significant interest to the cell biology field (Caviston and Holzbaur 2006; Soldati and Schliwa 2006; Akhmanova and Hammer 2010). The minus end–directed cytoplasmic dynein motor transports organelles and vesicles along microtubules to their proper subcellular locations, and defects in dynein function are causally linked to multiple neurodegenerative diseases (Perlson et al., 2010). Early endosomes are among the cargoes of the dynein motor, and dynein-mediated retrograde transport of Rab5-associated early endosomes is crucial for neuronal growth and survival (Delcroix et al., 2003). However, it is unclear how a Rab5-associated early endosome interacts with the dynein motor.

The dynactin complex is important for a variety of cytoplasmic dynein functions in vivo, including mitosis, vesicle transport, nuclear positioning, and spindle orientation (Schroer 2004; Kardon and Vale 2009), but whether it is involved in targeting the dynein motor to membranous cargoes has been a recent issue of debate (Haghnia et al., 2007). Within the dynactin complex, the Arp1 (actin-related protein 1) subunit forms an actin-like mini-filament of 37 nm, which is the backbone of the complex. One end of the Arp1 filament is associated with the barbed-end capping protein, and the other end binds to the pointed-end complex that contains Arp11, p62, p25, and p27 (Schafer et al., 1994; Eckley et al., 1999; Hodgkinson et al., 2005; Imai et al., 2006). The p150 subunit, together with p50 and p24, forms a shoulder/sidearm complex, which locates on the top of the Arp1 polymer. The p150 protein of dynactin increases dynein processivity (King and Schroer 2000; Culver-Hanlon et al., 2006; Kardon et al., 2009), and directly binds to the dynein intermediate chain (Karki and Holzbaur 1995; Vaughan and Vale 1995). Arp1 interacts with spectrin-like proteins, and thus, the Arp1 filament has been thought to link the dynactin complex and its associated dynein complex to membranous cargoes (Holleran et al., 1996, 2001; Muresan et al., 2001). Recently, spectrin mutations in Drosophila melanogaster have been shown to also cause defective axonal transport and neuronal degeneration (Lorenzo et al., 2010), which supports the importance of this interaction. However, a biochemical...
A previous study in Neurospora crassa (Steinberg and Schuster 2011). Defects in dynein and its regulators are important for nuclear distribution along the head of germ tubes. Our results show that there is no significant difference in the nuclear distribution patterns between wild type and the mutant (Fig. 1 D).

We then determined if p25 is required for dynein-mediated early endosome movement. In filamentous hyphae, dynein powers the minus end–directed movement of early endosomes (Steinberg and Schuster 2011). Defects in dynein and its regulators—dyactin and NUDF/LIS1—cause an abnormal buildup of early endosomes at the hyphal tip where microtubule plus ends locate (Lenz et al., 2006; Abenza et al., 2009; Zhang et al., 2010). In A. nidulans, RabA-associated early endosomes were observed using GFP-RabA and mCherry-RabA fusion proteins (Abenza et al., 2009; 2010). In wild-type cells, bidirectional movements of RabA-containing early endosomes were observed in time-lapse sequences (Video 1; Abenza et al., 2009).

In still images, early endosomes were seen to distribute along the hyphae (Fig. 2 A). In the Δp25 mutant, however, a very conspicuous, abnormal accumulation of mCherry-RabA-labeled early endosomes at the hyphal tip was seen (Fig. 2 A and

Results

Deletion of the A. nidulans p25 orthologue impairs movement of early endosomes but not nuclear distribution

We identified the p25 orthologue in the A. nidulans genome (An5022) by using the N. crassa p25 protein (Lee et al., 2001) as a query (http://www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html). A. nidulans p25 contains 202 amino acids with a predicted molecular weight of 21 kD. It shows significant sequence identity with p25 proteins from both N. crassa (197 amino acids, 21 kD; Lee et al., 2001) and mouse (182 amino acids, 20 kD; Eckley et al., 1999) with E-values of 7.6 × 10⁻⁴³ and 4.37375 × 10⁻⁴², respectively (Fig. 1 A). Sequence analysis suggests that p25 forms a left-handed β helix and contains hexapeptide repeats (Parisi et al., 2004). A. nidulans p25 also contains several characteristic hexapeptide repeats (Fig. 1 A).

We constructed a deletion allele of p25, Δp25, in A. nidulans (Fig. S1). Δp25 mutant colonies grown on plates appeared much healthier than ΔnudA (dynein heavy chain or HC) and ΔArp11 mutants, which exhibited a characteristic nud (nuclear distribution) colony phenotype characterized by small colony size and the lack of asexual spores (conidia; Fig. 1 B). The colony diameter of the Δp25 mutant was ~66% of the wild type, whereas those of the ΔnudA or ΔArp11 mutants were only ~15–20% of the wild type. Conidia (axial spores) were present on top of the colony as judged by the color of the colony (which comes from conidia) although conidiation (axial spore production) seemed less robust than in the wild type as the color of the colony is dimmer (Fig. 1 B). In contrast with loss-of-function mutations affecting the other dynactin components such as p150, Arp1, Arp11, p62, and p50, all of which produce a nud phenotype as indicated by clustering of multiple nuclei in the spore head of the germ tube (Zhang et al., 2003, 2008), nuclear distribution appeared normal in the Δp25 cells (Fig. 1 C).

A quantitative analysis of nuclear distribution was performed for wild type and the Δp25 mutant by counting the number of nuclei in the spore head of germ tubes. Our results show that there is no significant difference in the nuclear distribution patterns between wild type and the mutant (Fig. 1 D).

study showed that in Arp1-RNAi–treated Drosophila S2 cells, although dynactin cannot be fully assembled and vesicle transport is defective, dynein’s association with membrane compartments is not affected (Haghnia et al., 2007). Thus, it needs to be clarified whether Arp1 and its associated dyactin complex are important for targeting dynein to membranous cargoes.

In this study, we address the function of the dynactin complex in dynein–early endosome interaction in the filamentous fungus Aspergillus nidulans. In filamentous fungi, dynein and its regulators are important for nuclear distribution along elongated hyphae and also for the microtubule minus end–directed movement of early endosomes away from the tip (Morris 2000; Steinberg 2007; Peñalva 2010; Xiang and Oakley 2010).

A previous study in Neurospora crassa showed that among the dynactin components analyzed, p25 at the pointed end of the Arp1 filament is the only protein that is required for vesicle transport but not for nuclear distribution (Lee et al., 2001). Here we studied the role of p25 in early endosome transport, and our current results strongly suggest that p25 and its associated dynactin complex are important for dynein to interact with early endosomes.

Figure 1. The Δp25 mutant in A. nidulans does not exhibit a nud phenotype. (A) Protein sequence alignment of p25 proteins from A. nidulans (A.n.p25), N. crassa (N.c.p25; Lee et al., 2001), and mouse (Eckley et al., 1999). Identical amino acids are boxed in black. Blue lines indicate Hexapeptide repeats in A. nidulans p25 identified using the Simple Modular Architecture Research Tool (SMART) program. (B) The Δp25 mutant grows slightly more slowly than the wild type on plates but is much healthier than a typical nud mutant such as ΔnudA (dynein heavy chain or HC) or ΔArp11. (C) Unlike the ΔnudA and ΔArp11 mutants, the Δp25 mutant exhibits normal nuclear distribution. The strains were grown in liquid Y + UU medium for 7.5 h at 37°C before being fixed and stained with DAPI for visualizing the nuclei. Bars, 5 μm. (D) A quantitative analysis of nuclear distribution in the Δp25 mutant in comparison to that in a wild-type control strain. More than 200 germ tubes were analyzed for each strain. Means and standard deviations (error bars) were calculated from three experiments. No significant differences are revealed at the P-value of 0.05.
Interaction causes a low percentage (≤10%) of early endosomes to fall off the microtubule track at the plus ends but does not cause any significant accumulation of early endosomes at the hyphal tip (Schuster et al., 2011a,b). In Ustilago maydis, a 50% reduction of dynein accumulation at the plus ends caused by blocking EB1–dynactin interaction causes a low percentage (~10%) of early endosomes to fall off the microtubule track at the plus ends but does not cause any significant accumulation of early endosomes at the hyphal tip (Schuster et al., 2011a). Therefore, although the reduction of the A. nidulans HC’s plus-end accumulation in Δp25 cells may contribute to the endosome motility defect to certain extent, it cannot fully explain the dramatic accumulation of early endosomes at the hyphal tip and that the frequency of movements away from the hyphal tip in wild type cells is >10 fold of that in the Δp25 mutant (Fig. 2, A and B). This notion prompted us to further test the role of p25 in the interaction between dynein and early endosomes.

In NUDF/LIS1-depleted cells, p25 is required for the localization of dynein and dynactin to the hyphal tip region where early endosomes accumulate

In ΔnudF cells, GFP-HC forms a cloud-like structure at the hyphal tip where early endosomes also accumulate (Fig. 3 A and Videos 5–7; Zhang et al., 2010). Although the nature of this cloud-like hyphal tip accumulation of dynein is not clear, as it largely but not fully overlaps with the early endosome signals (Fig. 3 A and Videos 5–7), it most likely reflects dynein localization to the aggregate of early endosomes and/or other dynein cargoes accumulated in the same region. In alcA-nudF cells grown on glucose, a repressive condition that shuts off NUDF expression, GFP-HC and p150-GFP form cloud-like structures at a large majority (70% or more) of hyphal tips (Fig. 3, B and C; and Video 8). However, in the absence of p25, we have never found a single alcA-nudF cell that showed the cloud-like structure formed by GFP-HC and p150-GFP molecules at the hyphal tip, although comet-like structures are present and early endosomes are also accumulated at the hyphal tip in the same mutant background (Fig. 3, B–D). These results suggest that p25 is required for dynein/dynactin localization to the hyphal tip region where early endosomes and possibly other dynein cargoes are accumulated.
p25 and the dynactin complex are important for the physical interaction between the dynein complex and early endosomes

We developed a biochemical assay to determine if p25 and the dynactin complex are required for the physical interaction between early endosomes and dynein. We created strains coexpressing the S-tagged dynein intermediate chain (S-IC) and GFP-RabA. Cell extracts were prepared in the absence of detergent and incubated with S-protein agarose beads, which were washed gently and eluted with S-peptide as described previously (Zhuang et al., 2007). In the absence of detergent, a fraction of dynein molecules should be bound to early endosomes, and thus GFP-RabA, which specifically labels early endosomes, should be found in the eluate. To determine if the dynactin complex is important for dynein–early endosome interaction, we used extracts from alcA-nudF cells, both GFP-HC and mCherry-RabA localize to the hyphal tip region where early endosomes are found. Images of GFP-HC (B), p150-GFP (C), and GFP-RabA (D) are shown. In the absence of NUDF (alcA-nudF), a portion of GFP-HC and p150-GFP proteins localize to the hyphal-tip region, where early endosomes also accumulate (B–D, middle). Introducing the Δp25 mutant allele into this background (alcA-nudF/Δp25) abolishes this hyphal tip accumulation but maintains the plus-end comets of GFP-HC and p150-GFP (B and C, right). Bars, 5 µm.

Figure 3. In NUDF-depleted cells, p25 is required for the localization of dynein (GFP-HC) and dynactin (p150-GFP) to the hyphal tip region, where early endosomes are enriched. (A) Images showing that in the same nudF cells, both GFP-HC and mCherry-RabA localize to the hyphal tip region. (B–D) In NUDF-depleted cells (alcA-nudF grown on glucose), p25 is required for the localization of dynein and dynactin to the hyphal tip region where early endosomes are found. Images of GFP-HC (B), p150-GFP (C), and GFP-RabA (D) are shown. In the absence of NUDF (alcA-nudF), a portion of GFP-HC and p150-GFP proteins localize to the hyphal-tip region, where early endosomes also accumulate (B–D, middle). Introducing the Δp25 mutant allele into this background (alcA-nudF/Δp25) abolishes this hyphal tip accumulation but maintains the plus-end comets of GFP-HC and p150-GFP (B and C, right). Bars, 5 µm.

Loss of p25 does not significantly affect the Arp1 filament

Loss of p25 does not affect nuclear distribution, which suggests that the rest of the dynactin complex is intact in its absence. For example, Arp11 and p62 must be functional in the Δp25 mutant because loss of either of them prevents normal nuclear distribution (Lee et al., 2001; Zhang et al., 2008). In Arp11-depleted cells, S-tagged p150 pulled down significantly less Arp1, which suggests that in the absence of Arp11, the Arp1 filament consisting of multiple Arp1 subunits may be shortened and/or that the interaction between p150 and Arp1 is weakened (Fig. S2; Zhang et al., 2008). In marked contrast, in the Δp25 mutant the amount of pulled-down Arp1 was similar to that of the wild type (Fig. 5, A and B; and Fig. S2). Together, these results indicate that the effect of p25 deletion on dynein–early endosome interaction is unlikely to be caused by any significant alteration of the Arp1 filament. In addition, loss of p25 does not negatively affect dynein–dynactin interaction (Fig. S3), showing that the effect of p25 deletion on dynein–early endosome interaction is not caused by a loss of dynein–dynactin interaction.

Because loss of A. nidulans p25 does not significantly affect the integrity of the dynactin complex, we performed the following experiment to confirm that the A. nidulans p25 is indeed a component of the dynactin complex. We constructed a strain in which the expression of the p25–GFP fusion is under the control of the native p25 promoter and determined the localization of p25-GFP. As expected for a component of the dynactin complex, p25-GFP forms benomyl-sensitive comet-like structures (Fig. 5 C) resembling those previously seen with other dynein and dynactin components at the microtubule plus ends (Han et al., 2001; Zhang et al., 2003). Most interestingly, Western analyses showed that the p25-GFP protein levels are dramatically decreased in Arp1-depleted cells but not in p62-depleted cells (Fig. 5 D). This result suggests that the A. nidulans p25 depends on Arp1, rather than p62, to associate with the dynactin complex, and that in the mutant, we performed a Western blot analysis on the extracts used for the pull-down assay, and found that the protein levels of GFP-RabA are similar in the wild type and the alcA-Arp1 cells (Fig. 4 C). Together, these data strongly indicate that dynactin is important for linking dynein to early endosomes. We then determined the role of p25 in the physical interaction between dynein and early endosomes using the same assay. We used a strain containing the S-IC, Δp25, and GFP-RabA alleles to determine the levels of RabA pulled down by S-IC in the absence of p25. Compared with the wild type, the amount of pulled-down GFP-RabA proteins from the Δp25 mutant cell extract was significantly decreased (P < 0.001; Fig. 4, D and E). This decrease is not caused by the presence of lower levels of GFP-RabA in the mutant extract (Fig. 4 F). Thus, p25 plays an important role in the linkage between dynein and early endosomes. Our data also suggest that the extent of decrease in dynein–early endosome interaction caused by Δp25 is not significantly different from that caused by loss of Arp1 (at P = 0.05).
S-tagged p150 of dynactin is able to pull down p25-GFP from p62-depleted cell extracts as efficiently as from wild-type extracts (Fig. 5 E).
Discussion

Although dynein is known to be involved in the transport of a variety of membranous cargoes, how the motor is targeted to these cargoes is still a topic under investigation. In filamentous fungi and higher eukaryotic cells such as neurons, early endosomes undergo dynein-mediated transport, but how dynein is targeted to these vesicles is unclear (Akhmanova and Hammer 2010; Peňalva 2010). Our current data from the fungal model A. nidulans strongly suggest that p25 and its associated dynactin complex play a crucial role in linking dynein to early endosomes in vivo.

The dynactin complex physically interacts with the dynein complex through direct binding between p150 of dynactin and the dynein IC (Karki and Holzbaur 1995; Vaughan and Vallee 1995). It is thought that the dynactin complex facilitates dynein function by two different mechanisms. First, the p150 subunit of the dynactin complex enhances dynein processivity along microtubules, although whether this function is mediated via the microtubule-binding domain of p150 is a matter of dispute (King and Schroer 2000; Culver-Hanlon et al., 2006; Kim et al., 2007; Dixit et al., 2008; Kardon et al., 2009; Moore et al., 2009a). Second, the Arp1 filament within the dynactin complex is thought to mediate the physical interaction between dynactin and membranous cargoes, thereby targeting dynein to membranous organelles (Holleran et al., 1996, 2001; Muresan et al., 2001; Schroer 2004). However, this idea has been under debate recently, mainly because results from a study in Drosophila S2 cells argue against a role of Arp1 in dynein–membrane interaction (Haghnia et al., 2007). Although knocking down Arp1 using RNAi did impair the transport of membranous cargoes, it did not appear to affect dynein–membrane interaction as judged by a membrane-flotation assay (Haghnia et al., 2007). Because loss of Arp1 leads to disruption of the dynactin complex and also to a significant decrease in the level of the dynein-interacting p150 subunit both in Drosophila S2 cells and in filamentous fungi (Minke et al., 1999, 2007; Zhang et al., 2008), this study not only questions the role of Arp1 but also questions the role of the dynactin complex in targeting dynein to membranous cargoes.

Our current data strongly indicate the importance of the dynactin complex in linking dynein to early endosomes. In our biochemical experiments, we have examined specifically the association between dynein and early endosomes rather than the interaction of dynein with general membranous materials. This possibly explains the discrepancy between our conclusions and those of the earlier study in Drosophila S2 cells, in which the “general” dynein–membrane interaction was examined, as it is possible that dynactin is required for targeting dynein to some but not all the membranous cargoes. For example, the interaction between dynein and rhodopsin-carrying vesicles occurs in a dynactin-independent manner via the physical interaction between the Tctex-1 dynein light chain and the C-terminal cytoplasmic tail of rhodopsin (Tai et al., 1999). A recent study in Xenopus melanophores indicated that CLIP-170 at the microtubule plus end captures melanosomes in a dynactin-independent manner for their dynein-dependent minus end–directed transport (Lomakin et al., 2009). Previously, the C-terminal region of p150Glued dynactin has been implicated in the interaction between dynein and Rab7-marked late endosomes (Johansson et al., 2007), but a recent study indicates that in neuronal cells the direct interaction between dynein intermediate chain and Snapin is important for late endosome movement (Cai et al., 2010). In addition, the interaction between dynein and Rab11-containing recycling endosomes seems to be mediated by the dynein light intermediate chain (Horgan et al., 2010), and the dynein LC8 light chains are responsible for linking dynein to Piccolo-Bassoon transport vesicles (Fejtova et al., 2009). It is also interesting to note that the mitotic checkpoint protein ZW10 recruits dynein not only to kinetochores, but also to ER-Golgi membranes (Vallee et al., 2006). This recruitment may be mediated by the ZW10-dynamitin (p50 of dynactin) interaction as indicated by yeast two-hybrid analysis (Starr et al., 1998), or by a direct interaction between ZW10 and phosphorylated dynein intermediate chain, which recruits dynein to kinetochores before metaphase (Whyte et al., 2008). Finally, interaction between dynein and the membranous spindle matrix is mediated by a dynein-interacting protein Nudel (Ma et al., 2009). Given the variety of targeting mechanisms used by different populations of vesicles and membranous structures, it is important to address specifically the interaction of dynein with any specific class of membranous organelles.

Our current work not only demonstrates the importance of the dynactin complex in targeting dynein to early endosomes, but it also uncovers a specific role of the p25 component of dynactin in dynein–early endosome interaction. In a previous study in N. crassa, p25 was found not to be required for the interaction between dynein and membranes, and indeed deletion of p25 appeared to strengthen dynein–membrane interaction (Lee et al., 2001). Because a general membrane-flotation assay was used in the N. crassa study, whereas a specific dynein–early endosome interaction assay was used in our study, we think that, as discussed earlier, p25 is required for dynein to interact with a subset but not all membranous vesicles. It should be pointed out that although our study points out an important role of p25 in dynein–early endosome interaction, our data do not exclude the possibility that p25 may cooperate with other dynactin components, such as Arp1, to interact with early endosomes. Because different dynactin components affect each other within the complex, it is not straightforward to use knockout or knockdown methods to address the specific roles of each dynactin component in the dynein–early endosome interaction. For example, the Arp1 protein is the backbone of the dynactin complex, and its loss leads to a disruption of the whole complex. In Drosophila and in filamentous fungi such as N. crassa and A. nidulans, loss of Arp1 results in a dramatic decrease in the protein level of p150 (Minke et al., 1999, 2007; Zhang et al., 2008), the key component of dynactin that mediates the interaction between dynein and dynactin (Karki and Holzbaur 1995; Vaughan and Vallee 1995). In addition, loss of other Arp1 pointed-end proteins such as Arp11 and p62 significantly lowers the levels of Arp1 pulled down by S-tagged p150, which suggests that these proteins are required for the integrity of the Arp1 filament and/or for the p150–Arp1 interaction. Moreover, loss of function of many dynactin components, such as p150,
Arp1, Arp11, p62, and p50, causes a dramatic defect in dynein localization to the microtubule plus ends, as dynein comets are either completely absent or extremely hard to be observed in the mutants (Zhang et al., 2003; Lenz et al., 2006; Zhang et al., 2008), which should lead to a defect in dynein–early endosome interaction. However, loss of p25 does not apparently disrupt the function of the core complex because nuclear distribution is completely normal in the p25-null mutant. The idea that the absence of p25 does not apparently affect either the Arp1 filament or other pointed-end proteins is also supported by the results of our biochemical experiments. Although loss of Arp11 or p62 significantly reduced the amount of Arp1 pulled down by p150 (Zhang et al., 2008), loss of p25 does not produce this effect. Although we would not exclude the possibility that p25 may affect the dynactin complex in a subtle way that may contribute to the defect of dynein–early endosome interaction, our data strongly support the conclusion that p25, when associated with the dynactin complex, plays a significant role in dynein–early endosome interaction.

In the vertebrate dynactin complex, p25 forms a complex with p27, Arp11, and p62 at the pointed end of the Arp1 filament (Schoeber 2004). It is interesting to note that some or all of these four pointed-end components may be missing in some eukaryotic organisms. For example, Saccharomyces cerevisiae does not contain obvious orthologues of p25, p27, and p62 (Eckley et al., 1999; Moore et al., 2008), which is consistent with the known function of S. cerevisiae dynein in nuclear migration/spindle orientation but not vesicle transport (Yeh et al., 2004). As the mouse Arp11 protein shows higher homology with other Arps in the S. pombe genome than with the candidate Arp10 A. nidulans and Arp10 in S. cerevisiae have similar functions in regulating the Arp1 filament (Moore et al., 2008). All these pointed-end proteins are present in N. crassa (Borkovich et al., 2004), U. maydis, and Dictyostelium discoideum. It would be important to investigate and compare the functions of these pointed-end proteins in the transport of various organelles in a variety of organisms.

**Materials and methods**

**Strains and media**

A. nidulans strains used in this study are listed in Table 1. For biochemical experiments involving dynactin isolation, YG (yeast extract plus glucose) + UU (or YUU) liquid medium was used. For DAPI staining of nuclei, YLU liquid medium was used. For live cell imaging experiments, minimal medium containing glucose or glycerol plus supplements was used.

**Construction of the p25 deletion mutant**

The p25 deletion (Δp25) construct was made using a fusion PCR strategy as described previously (Szewczyk et al., 2006). Specifically, the upstream region was amplified from wild-type genomic DNA using P25u5′ (5′-CAACGAGTACAACATCTC-3′) and P25u3′ (5′-TGTCTTGGCCGCAATTTG-3′), and the downstream region was amplified using P25d5′ (5′-ATCCGAGTCTAATAC-3′) and P25d3′ (5′-AGATACGCCATACGCGAC-3′). Apergillus pyrG was used for replacing the coding region. The p25 was amplified from the plasmid pAO81 (Yang et al., 2004) using ApyrG5′ (5′-CTATCGAATTCGGGCGGCATAAGCATGCTTCTCCACT-3′) and ApyrG3′ (5′-AATCCTAAGTGACATCTCCGATTGAGGAGGACC-3′). The final linear Δp25 construct obtained by fusion PCR was transformed into the LZ12 strain that contains GFP-dynein HC under the control of its native promoter (Zhang et al., 2007). Transformants were screened using PCR of genomic DNA to confirm the correct integration of the construct into the p25 locus. The primers used for verifying the correct integration were p25UU (p1; 5′-CGGAAAAGATGTCGACG-3′), ApyrG3′ (p2; 5′-GTGTCGCGTGGAGGTATT-3′), ApyrG5′ (p3; 5′-AGCAATGTCGACATACG-3′) and p25DD (p4; 5′-AACAGAAAAATGGAACAC-3′). The result of the PCR analysis is shown in Fig. S1, p1 and p4 are not located within the flanking sequences of the Δp25 construct but just upstream and downstream of the sequences [Fig. S1 A]). The 1 kb (with p1 + p2) and 1.1 kb (with p3 + p4) products were specifically produced using the genomic DNA isolated from the Δp25 mutant as template, which indicates that the integration occurred in a site-specific manner as expected [Fig. S1 B]. The site-specific integration was also confirmed by a Southern blot analysis on XhoI-digested genomic DNAs using the 3′ flanking sequence of the Δp25 construct as a probe [Fig. S1, C and D]. Moreover, the absence of other unexpected signals beside the 2.6 kb expected signal on the Southern blot suggests that the Δp25 construct only underwent a site-specific integration into the p25 locus in the Δp25 mutant strain [Fig. S1, C and D].

**Construction of the p150-GFP strain**

A C-terminal region of the nudM open reading frame (ORF) and the 3′ untranslated region (UTR) were amplified from wild-type genomic DNA, with corresponding primer pairs of ORF forward (5′-GGGATCCACTACACGTTGGAGTACAACCTG-3′) and ORF reverse (5′-AAATTGACGTTTTAATGCTTCT-3′), and UTR forward (5′-TTACCGTCATGAAGGCGACG-3′) and UTR reverse (5′-TTACCGTCATGAAGGCGACG-3′). The DNA containing GFP and an AfpyrG gene was amplified from the pNO3 plasmid (deposited to the Fungal Genetics Stock Center by Stephen A. Osmani, Ohio State University, Columbus, OH; Yang et al., 2004; McLuskey et al., 2010) with the fusion primer forward (5′-CAGACCTTGGACGGAGCATAAAAACACCTAAGGACGCTGAG-3′) and the reverse primer reverse (5′-GATGGATCCCTTTAGGCGGCATTTTG-3′). The fusion product was then fused to the UTR after sequences encoding almost the entire linker (GGAGCTGGTGCAGGC-3′) and UTR-R2 (5′-CAGCCTGTTGAGCGAGCAATTAAACCAACCTTAGGAGCTG-3′) and the fusion reverse primer (5′-AGAGTACACTATCTC-3′). The resultant PCR product, containing GFP and AfpyrG gene, was amplified from the pNO3 plasmid (deposited to the Fungal Genetics Stock Center by Stephen A. Osmani, Ohio State University, Columbus, OH; Yang et al., 2004; McLuskey et al., 2010) with the fusion primer forward (5′-CAGACCTTGGACGGAGCATAAAAACACCTAAGGACGCTGAG-3′) and the reverse primer reverse (5′-GATGGATCCCTTTAGGCGGCATTTTG-3′). The fusion product was then fused to the UTR after another fusion PCR using ORF-F2 (5′-CTCTCCATCGAAATGTTCT-3′) and UTR-R2 (5′-GATGGATCCCTTTAGGCGGCATTTTG-3′). The fusion fragment was transformed into the TNO2A3 strain (provided by B. Oakley, University of Kansas, Lawrence, KS; Nayak et al., 2006). Transforms...
were selected by microscopic observation of the microtubule plus-end comets, followed by Western analysis using a GFP antibody (Covance) and the p150 antibody (Zhang et al., 2008).

### Construction of the p25-GFP strain

Construction of the p25-GFP fusion was done similarly to the construction of the p150-GFP fusion described above, except that the following primers were used. p25 ORF forward (5'-ATGCCATATGCCCTCCGGC-3'), p25 ORF reverse (5'-TCGGGAGAGATATCGAAGTATCCGAGGAGCTGGTGCAGGCGCT-TCTGAGAGGAGGCACTGATG-3'), and p25 UTR reverse (5'-AGATCTGGTACCTAGCCGC-3'), and p25 UTR reverse (5'-AGATCTGGTACCTAGCCGC-3'), and p25 UTR reverse (5'-AGATCTGGTACCTAGCCGC-3'). The fusion fragment was transformed into the TN02A3 strain (Nayak et al., 2006). Transformants were selected by microscopic observation of the microtubule plus-end comets, and presence of the p25-GFP fusion gene in the genome was verified by PCR analyses.

### Construction of the GFP-RabA strain in which the GFP-RabA fusion is driven by the constitutive gadA<sup>+</sup> promoter

We first constructed the plasmid p1989 containing the gadA<sup>+</sup>-gfp-rabA<sup>lma</sup> fusion gene linked to the A. nidulans pyroA gene. For making the plasmid, two primers—JFA190 5'NslnRaba and JFA191 3'Xmol-raba—were synthesized. JFA190 5'Nsln-raba (5'-ATGCCATATGCCCTCCGGC-CATCG-3') contains a restriction site for NsiI, and a substitution of the second codon of raba (CAT is used to replace the original GTC for creation of the restriction site). JFA191 3'Xmol-raba (5'-CCCGGGAATTCAGAATCCGGCAATCCGGC-3') contains a restriction site for XmaI (or S-IC); 89 Zhang et al., 2008).

These two primers were used to amplify the rabA-coding region from a pGEM plasmid that contains the cDNA sequence of raba, and the product was digested with the restriction enzymes NsiI and XmaI. The digested fragment was cloned into the NsiI-XmaI restriction sites in the plasmid carrying the gadA<sup>+</sup>-gfp fusion upstream of these sites and a gadA terminator downstream of these restriction sites, as well as the A. nidulans pyroA gene as a selection marker. The resulting plasmid, p1989, was used to transform A. nidulans strain MAD2743. A. nidulans strain MAD2743 was selected from the transformants for further studies because a Southern blot analysis on BamH1-digested genomic DNA indicates that this strain contains a single-copy integration of the plasmid at the pyroA locus.

### Live cell imaging

Cells were grown at 32°C overnight in 0.5 ml of minimal medium with glycerol or glucose plus supplements using the Laboratory-Tek Chambered #1.0 Borosilicate Coverglass System. Images were captured at room temperature using an inverted fluorescence microscope (IX70; Olympus) with a 100x objective lens (numerical aperture, 1.35; Olympus). A cooled charge-coupled device camera (Sensicam QE; PCO/Cooke Corporation) was used. A filter wheel system with GFP/mCherry-ET Sutterted series with high transmission (BioVision Technologies) was used. The IPLab software was used for image acquisition and analysis. For GFP and mCherry images, a 100-ms exposure time was used. For measuring the signal intensity of the individual GFP/HC comets, an area containing the whole comet was selected as a region of interest (ROI), and the Max/Min tool of the IPLab program was used to measure the maximal intensity within the ROI. Then the ROI box was dragged outside of the cell to take the background value, which was then subtracted from the value of the comet.
comets that have arrived at the hyphal tip show the highest signal intensity, as we have described previously (Zhang et al., 2003; Efimov et al., 2006). We used selected frames within a sequence in which the comets are seen at the hyphal tip region, and only those comets that have arrived at the hyphal tip were measured. GFP-HC signals in wild type and in the Δp25 mutant were measured under the same conditions. Photoshop (Adobe) was used to process images after data acquisition. Images in Fig. 2 and Fig. 3 were processed in Photoshop to increase the brightness (wild-type and mutant images within one figure were always treated together rather than individually) so that cell shape can be seen more easily.

Biochemical analyses of dynine–early endosome interaction, p150–Arp1 interaction, and dynine–dynactin interaction

To study dynine–early endosome interaction, we took advantage of the previously constructed strains expressing SIC (Zhuang et al., 2007), and determined if dynine can pull down GFP-RabA–associated early endosomes in the absence of detergent. We modified the previously made GFP-RabA construct so that the fusion is driven by the constitutive gpdA promoter instead of the inducible alcA promoter (Abenza et al., 2009; Pantazopoulou and Peñalva 2009), which allows the GFP–RabA fusion to be expressed in regular rich medium instead of minimal medium with glycerol as a carbon source. This modification facilitated the biochemical analyses because cells grow much more robustly in rich medium than in glycerol-containing minimal medium. Strains containing both STagged dynine IC (Zhuang et al., 2007) and GFP-RabA were constructed by genetic crossing. 80 g of hyphal mass after an overnight culture was used for each experiment. Cell extracts were incubated with S-agarose instead of the inducible gpdA promoter to remove cell debris. The extracts were incubated with S-agarose beads, followed by gentle washing and then elution with 10 mg/ml of 2007) and GFP-RabA were constructed by genetic crossing. 8 g of hyphal mass after an overnight culture was used for each experiment. Cell extracts were incubated with S-agarose instead of the inducible gpdA promoter to remove cell debris. The extracts were incubated with S-agarose beads, followed by gentle washing and then elution with 10 mg/ml of 2007) and GFP-RabA were constructed by genetic crossing. 8 g of hyphal mass after an overnight culture was used for each experiment. Cell extracts were incubated with S-agarose instead of the inducible gpdA promoter to remove cell debris. The extracts were incubated with S-agarose beads, followed by gentle washing and then elution with 10 mg/ml of 2007) and GFP-RabA were constructed by genetic crossing. 8 g of hyphal mass after an overnight culture was used for each experiment. Cell extracts were incubated with S-agarose instead of the inducible gpdA promoter to remove cell debris. The extracts were incubated with S-agarose beads, followed by gentle washing and then elution with 10 mg/ml of

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Online supplemental material

Fig. S1 shows site-specific integration of the Δp25 mutant as indicated by PCR and Southern blot analyses. Fig. S2 shows that STagged p150 (S-p150) pulled down a normal amount of Arp1 from Δp25 cell extract but not from alcA-Arp1 cell extract. Fig. S3 shows that dynine–dynactin interaction is not negatively affected by loss of p25. Video 1 shows bidirectional movements of early endosomes labeled by mCherry-RabA in a wild-type hypha. Videos 2 and 3 show that early endosomes in a ΔnudA (dynine heavy chain) hypha are largely accumulated at the hyphal tip. Video 4 shows that early endosomes in a ΔnudA (dynine heavy chain) hypha are largely accumulated at the hyphal tip. Video 5 shows GFP-dynein HC (GFP-HC) signals in the ΔnudA mutant. Video 6 shows mCherry-RabA signals in the same ΔnudA cells shown in Video 5. Video 7 shows a merge of the GFP-HC and mCherry-RabA signals in the same ΔnudA mutant cells as shown in Videos 5 and 6. Video 8 shows a cloud-like structure at the hyphal tip formed by p150-GFP in ΔalcAΔnudC cells.

Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201011022/DC1.


Figure S1. **Site-specific integration of the Δp25 construct into the genome of the Δp25 mutant as indicated by PCR and Southern analyses.** (A) A diagram showing the Δp25 linear construct with the ApyrG marker flanked by the 5' and 3' flanking sequences of the p25 gene. Homologous recombination events that occurred between this construct and the wild-type genome (wild type) are indicated by crosses. The resulting Δp25 locus is shown at the bottom. The positions of the primers used for PCR analyses are indicated by arrows. Note that these primers are located outside of the flanking sequences of the Δp25 construct and thus will produce the expected products of 1.0 kb and 1.1 kb only when the construct integrated into the p25 locus. (B) Result of a PCR analysis on genomic DNAs from the Δp25 mutant and a wild-type strain (WT). (C) A diagram showing the sizes of expected signals on a Southern blot using XhoI-digested genomic DNAs from wild type and the Δp25 mutant when the 3' flanking region of the Δp25 construct is used as a probe. A 4.8-kb band is expected from the wild type based on the genomic sequence of the region. However, because a XhoI site is within the ApyrG marker, a 2.6 kb signal is expected from the Δp25 mutant. (D) The result of the Southern analysis shows expected signals both in the wild type (WT) and the Δp25 mutant samples.

Figure S2. **S-tagged p150 (S-p150) pulled down a normal amount of Arp1 in extract from Δp25 cells but not from alcA-Arp11 cells.** To test whether Δp25 affects p150–Arp1 interaction, the alcA-Arp11 mutant was used as a positive control, as we showed previously that loss of Arp11 negatively affected the amount of Arp1 pulled down by the S-tagged p150 (Zhang et al., 2008). In addition, different loadings of the wild-type sample on the same gel were done, which strengthens our conclusion that loss of Arp11 significantly lowered the amount of Arp1 pulled down by S-p150 (Zhang et al., 2008). However, loss of p25 does not apparently affect the amount of Arp1 pulled down by S-p150. WT-1, 20 µl; WT-2, 30 µl; WT-3, 40 µl. For both thealcA-Arp11 and Δp25 mutants, 40 µl of eluate was loaded.
Figure S3. **Dynein–dynactin interaction is not negatively affected by loss of p25.** (A) In the extracts of the Δp25 mutant, S-p150 pulled down dynein heavy chains, and the amount of pulled-down dynein was even higher than that in the extract of wild-type cells. (B) A quantitative analysis of the Western results. All the values were relative to wild-type values, which were set as 1. Mean and standard deviation values (error bars) were based on results from three independent pull-down experiments (P < 0.05). (C) Western analyses on total extracts indicate that the relatively high level of pulled-down dynein in the Δp25 mutant is not caused by a relatively high level of total dynein in the mutant. A similar phenomenon was also observed during our previous study on Arp11 (Zhang et al., 2008). Although we currently do not understand the mechanism behind this phenomenon, the fact that nuclear distribution appears normal in the Δp25 mutant suggests that this enhancement in dynein–dynactin interaction does not significantly affect the normal function of dynein.

**Video 1.** The mCherry-RabA fusion protein is expressed in a wild-type strain, which allows early endosomes to be labeled. By using an inverted fluorescence microscope (IX70; Olympus), early endosomes were observed to undergo bidirectional movements. 30 frames were taken with a 0.1-s exposure time and a 0.3-s interval between frames. The movie has been sped up 5 times.

**Video 2.** Early endosomes labeled by mCherry-RabA in a Δp25 hypha are largely accumulated at the hyphal tip. Note that an early endosome moves toward the hyphal tip. An inverted fluorescence microscope (Olympus IX70) was used for capturing images. 30 frames were taken with a 0.1-s exposure time and a 0.3-s interval between frames. The movie has been sped up 5 times.

**Video 3.** Early endosomes labeled by mCherry-RabA in a Δp25 hypha are largely accumulated at the hyphal tip. This is a different hypha than that in Video 2. Note that many early endosomes along the hypha do not undergo directional movements. An inverted fluorescence microscope (IX70; Olympus) was used for capturing images. 12 frames were taken with a 0.1-s exposure time and a 1-s interval between frames. The movie has been sped up 10 times.

**Video 4.** Early endosomes labeled by mCherry-RabA in a ΔnudA (dynein heavy chain) hypha are largely accumulated at the hyphal tip. An inverted fluorescence microscope (IX70; Olympus) was used for capturing images. 12 frames were taken with a 0.1-s exposure time and a 1-s interval between frames. The whiteness of the images was enhanced to show the nonmobile mCherry-RabA positive dots in the hypha. The movie has been sped up 10 times.
Video 5. **GFP-dynein HC (GFP-HC) signals in the ∆nudF mutant.** The GFP-HC fusion was introduced into the ∆nudF mutant by a genetic cross. An inverted fluorescence microscope (IX70; Olympus) was used for capturing images. 12 frames were taken with a 0.1-s exposure time and a 1-s interval between frames. The movie has been sped up 10 times.

Video 6. **mCherry-RabA signals in the same ∆nudF cells shown in Video 5.** The mCherry-RabA fusion was introduced into the ∆nudF mutant containing GFP-HC by a genetic cross. An inverted fluorescence microscope (IX70; Olympus) was used for capturing images. 12 frames were taken with a 0.1-s exposure time and a 1-s interval between frames. The movie has been sped up 10 times.

Video 7. **Merge of the GFP-HC and mCherry-RabA signals in the same ∆nudF mutant cells as shown in Videos 5 and 6.** Note that both GFP-HC and mCherry-RabA localize to the hyphal tip region, and the signals largely but not fully overlap with each other. An inverted fluorescence microscope (IX70; Olympus) was used for capturing images. 12 frames were taken with a 0.1-s exposure time and a 1-s interval between frames. The movie has been sped up 10 times.

Video 8. **A cloud-like structure at the hyphal tip formed by p150-GFP in alcA-nudF cells.** The p150–GFP fusion was introduced into the alcA-nudF mutant by a genetic cross. An inverted fluorescence microscope (IX70; Olympus) was used for capturing images. 12 frames were taken with a 0.1-s exposure time and a 1-s interval between frames. The movie has been sped up 10 times.

Reference