Identification of a novel site in the tail of dynein heavy chain important for dynein function in vivo

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Running title: A novel site in the dynein tail is important in vivo

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Key words: cytoplasmic dynein; Aspergillus nidulans; microtubule motor

Background
The dynein heavy chain tail is required for subunit interactions but not for in vitro motility.

Results
A dynein tail mutation affects dynein function in vivo without affecting subunit interactions.

Conclusion
A site upstream of the subunit interaction sites of the dynein tail is important in vivo.

Significance
This study discovers a novel site of the dynein tail critical for motor function in vivo.

Summary
The minus-end-directed microtubule motor cytoplasmic dynein is responsible for the intracellular movements of many organelles including nuclei and endosomes. The dynein heavy chain (HC) contains a C-terminal motor domain and an N-terminal tail domain. The tail binds other dynein subunits and the cargo-interacting dynactin complex, but is dispensable for movement of single dynein molecules in vitro. Here we identified a mutation in the Aspergillus nidulans HC tail domain, nudA<sup>F208V</sup>, which causes obvious defects in dynein-mediated nuclear positioning and early endosome movement. Astonishingly, the nudA<sup>F208I</sup> mutation in the same position does not cause the same defects, suggesting that a subtle difference in the size of the amino acid side chain at this position has a significant consequence. Importantly, our biochemical analyses indicate that the nudA<sup>F208V</sup> mutation does not affect dynein subunit interactions and the mutant dynein is also able to bind dynactin and another dynein regulator, NUDF/LIS1. The mutant dynein is able to physically interact with the early endosome cargo, but dynein-mediated early endosome movement away from the hyphal tip occurs at a significantly reduced frequency. Within the small group of early endosomes that move away from the hyphal tip in the mutant, the average speed of movement is lower than that in the wild type. Given the dispensability of the dynein tail in dynein motility in vitro, our results support the notion that the structural integrity of the dynein tail is critical in vivo for the coordination of dynein force production and movement when the motor is heavily loaded.

Introduction
Cytoplasmic dynein, a minus-end-directed microtubule motor, is important for a variety of cellular functions including organelle positioning, and the intracellular transport of vesicles, proteins and viruses (1-6). Defects in cytoplasmic dynein and its regulatory proteins such as dynactin (7), or LIS1 (8), cause neurodegenerative and neurodevelopmental disorders (8,9). The dynein heavy chains (HCs) within the dynein complex form a dimer that contains motor activity responsible for ATP-dependent movement of dynein along microtubules. Each HC contains a C-terminal motor domain that forms a ring containing six AAA (ATPase of various cellular activities) domains and a microtubule-binding stalk located in between AAA4 and AAA5 (10-13). The N-terminus of the HC is called the “tail” (or the “stem”) that is implicated in dynein HC dimerization and binding to other subunits including intermediate chains (ICs), light...
intermediate chains (LICs) and light chains (LCs) (14). The dynein IC contains binding sites for the LCs, dynactin and another dynein regulator NudE that recruits LIS1 (14-26). Although the linker region immediately before the AAA1 domain is involved in power stroke, the HC tail N-terminal to the subunit-interaction site is not important for movements of single dynein molecules in vitro (27-31). It has been proposed, however, that the tail plays a role in regulating dynein motor function (32). This hypothesis is supported by the results that the neurodegenerative mutation Loa (Legs at odd angels, F580Y in mouse HC) in the HC tail affects dynein motor processivity (32), or velocity (33). However, since defects in dynein subunit interactions and/or interaction with dynactin have been detected in the Loa mutant (32, 34), the contribution of other factors to the Loa phenotype is hard to be completely excluded.

The filamentous fungus Aspergillus nidulans is an excellent genetic system for studying intracellular trafficking and the regulation of cytoplasmic dynein (35-38). In A. nidulans, components of the dynein/dynactin complexes and other regulators including LIS1 and NudE/Nudel are required for nuclear distribution (nud), and loss of function of these proteins produces a nud phenotype (19, 36, 39). Beside its function in nuclear distribution, dynein in filamentous fungi is also critical for the retrograde movement of early endosomes in hyphae as first discovered in Ustilago maydis (35-38, 40). In A. nidulans, as well as in Saccharomyces cerevisiae, U. maydis and mammalian cells, dynein forms comet-like structures, representing their accumulation at the microtubule plus ends (40-44). In A. nidulans and U. maydis, this accumulation at the microtubule plus ends near the hyphal tip facilitates dynein-early-endosome interaction, and thus is important for dynein-driven transport of early-endosomes from the hyphal tip (37, 40, 45-47). The plus-end accumulation of dynein depends on the integrity of the dynein-dynactin complexes and kinesin-1 (40, 48-53). Loss of IC abolishes plus-end comet formation (48), presumably because dynein could no longer bind to p150 dynactin (15-17), a key factor in A. nidulans dynein’s plus-end localization (52, 53). Loss of LIC also abolishes plus-end comet formation because HC-IC interaction is significantly weakened in the absence of LIC (50).

In this current study, we have identified a HC tail mutation that does not affect the accumulation of dynein at the microtubule plus ends but causes obvious defects in dynein-mediated early endosome transport and nuclear migration. This mutation, nudA<sup>F208V</sup>, is far upstream from the previously mapped IC- and LIC-binding site, and our experimental data demonstrate that it does not affect dynein complex formation or dynein-early-endosome interaction. Interestingly, changing the valine residue to isoleucine completely rescues the mutant phenotype, suggesting that a subtle change in the size of the side chain of this residue is sufficient for causing a significant effect on HC function in vivo. Thus, beyond HC tail’s known role in interacting with other dynein subunits implicated in cargo binding, a novel site upstream of the subunit interaction site plays a significant role in dynein motor function in vivo.

**Experimental procedures**

*A. nidulans* strains, media and mutagenesis

*A. nidulans* strains used in this study are listed in Table 1. For biochemical experiments, YG (yeast extract plus glucose) + UU (or YUU) liquid medium was used. UV mutagenesis on spores of *A. nidulans* strains was done as previously described (54, 55). For DAPI staining of nuclei, cells were incubated in YUU medium for 8 hours at 37°C. For live cells imaging experiments, liquid minimal medium plus supplements was used, and cells were cultured at 32°C for overnight and observed at room temperature.

For observing early endosome movement in vivo, we used a minimal medium containing either 1% glycerol (v/v) or 0.9% (w/v) fructose and 6.25 mM threonine (56). For observing GFP-HC, we also used these two types of minimal media, and in addition, we used a minimal medium containing either 0.1% (w/v) glucose or 1% glucose.

**Identification of the nudA<sup>F208V</sup> mutation and creation of the nudA<sup>F208I</sup> mutation**

The nudA sequence of the 4-63 mutant corresponding to the wild type nudA fragment that complemented the 4-63 mutant was sequenced using primers as previously described (57), which
led to the identification of the nudA$^{F208V}$ mutation. To construct the mutant strain carrying the nudA$^{F208I}$ mutation, we first made the DNA fragment containing this mutation using fusion PCR. Specifically, two fragments were made using the primer pairs of NudA51 (AAAACTCTATCTGCCCGAA) and FIR (GAGTTCCAGCTCCGCATTCTCTTTAGTG GCC), and of NudA33 (CAATGGAAATCTGATA ACGCG) and FIF (GGCACTAAGAATAATCGCCGAGCTGG AACCT) respectively, followed by a fusion PCR using the primer pair of NudA51 and NudA33. This fragment was transformed into the RQ8 strain containing the nudA$^{F208V}$ mutation, and wild-type-looking transformants were subjected to sequencing analysis.

Identification of suppressors of the nudA$^{F208V}$ mutation

We performed a UV mutagenesis on the RQ8 strain containing the nudA$^{F208V}$ mutation. UV mutagenesis on spores of the RQ8 strain was done as previously described (54, 55). Colonies that looked healthier than that of the nudA$^{F208V}$ mutant were judged to be suppressors and selected from the plates. Two suppressors were selected and crossed with a wild-type strain. We did not see any nudA$^{F208V}$-like colonies among the progeny, suggesting that the suppressor mutations are linked to the original nudA$^{F208V}$ mutation. These suppressors were further analyzed by sequencing. Primers for sequencing were as described previously (57).

Live cell image analyses

Fluorescence microscopy of live A. nidulans hyphae was as described (47, 52). Cells were grown at 32°C overnight using the Lab-Tek chambered coverglass system. Images were captured at room temperature using an Olympus IX70 inverted fluorescence microscope (with a 100x objective) linked to a PCO/Cooke Corporation Sensicam QE cooled CCD camera. A filter wheel system with GFP/mCherry-ET Sputtered series with high transmission (Biovision Technologies) was used. The IPLab software was used for image acquisition and analysis.

Analyses of protein-protein interactions, sucrose gradient centrifugation and western blotting

Dynein HC-IC interactions were examined using strains containing a functional S-tagged dynein IC (S-IC) as done previously (57). Alternatively, the μMACS GFP isolation kit (Miltenyi Biotec) was used to determine if GFP-tagged dynein HC pulls down IC. About 0.7 g hyphal mass was harvested from overnight culture for each sample, and cell extracts were prepared using a lysis buffer containing 25 mM Tris-HCl, pH 8.0, 0.4% Triton X-100, 0.3% (v/v) of a protease inhibitor cocktail (Sigma-Aldrich) and 0.1% (v/v) of a phosphatase inhibitor cocktail (Sigma-Aldrich). Cell extract was centrifuged at 16,000 g for 30 min. at 4°C, and supernatant was used for the pull-down experiment. To pull down GFP-tagged protein, 50 μL anti-GFP MicroBeads were added into the cell extract and incubated on ice for 30 min. The MicroBeads/cell extracts mixture was then applied to a μColumn followed by gentle wash with the lysis buffer. After four times washing with the lysis buffer, the column was finally washed one more time with the lysis buffer without any detergent. The Pre-heated (95°C) SDS-PAGE sample buffer was used as elution buffer. For analyzing dynein complex sedimentation using sucrose gradient centrifugation, the cell extract was centrifuged at 100,000 g and the supernatant was loaded on the top of a 5-25% sucrose step gradient and centrifuged at 4°C overnight at 100,000 g using the SW41 rotor. Fractions were collected from the top and analyzed on protein gels. Antibodies against dynein HC, dynein IC, p150 dynactin and NUDF/LIS1 were described previously (39, 51, 58). Anti-GFP antibodies from Covance (monoclonal) and Clontech (polyclonal) were also used for western analyses. Western analyses were performed using the alkaline phosphatase system and blots were developed using the AP color development reagents from Bio-Rad.

Analyses of dynein-early-endosome interaction

For the biochemical analyses of dynein-early-endosome interaction, we used the strains containing GFP-labeled dynein HCs and mCherry-RabA-labeled early endosomes. The strains were grown overnight in the liquid minimal medium containing 0.9% (w/v) fructose and 50 mM...
threonine, which induces the expression of the mCherry-RabA fusion under the control of the alcA promoter. About 0.7g hyphal mass was harvested from overnight culture for each sample, and cell extracts were prepared using a lysis buffer containing 25 mM Tris-HCl, pH 8.0, 0.01% Triton X-100, 0.3% (v/v) of a protease inhibitor cocktail (Sigma-Aldrich) and 0.1% (v/v) of a phosphatase inhibitor cocktail (Sigma-Aldrich). Cell extract was centrifuged at 4,000 g for 30 min. at 4°C, and supernatant was used for the pull-down assay using the μMACS GFP isolation kit (Miltenyi Biotec). As described earlier, 50 μL anti-GFP MicroBeads were added into the cell extract and incubated on ice for 30 min. The MicroBeads/cell extracts mixture was then applied to μColumn followed by gentle wash with the lysis buffer five times. Pre-heated (95°C) SDS-PAGE sample buffer was used as elution buffer. The anti-mCherry antibody used on the western blots to detect mCherry-RabA was purchased from BioVision Research Products. Western analyses were performed using the alkaline phosphatase system and blots were developed using the AP color development reagents from Bio-Rad. Quantification of the protein band intensity was done using the IPLab software as described previously (52), with a minor modification. Specifically, an area containing the whole band was selected as a region of interest (ROI), and the intensity sum within the ROI was measured. Then the ROI box was dragged to the equivalent region of the negative control lane to take the background value, which was then subtracted from the intensity sum. The intensity ratio of the pulled-down mCherry-RabA to GFP-HC was calculated. The ratios calculated from the wild-type samples were set as 1, and the relative ratios of the mutant were calculated and presented.

Results

The nudA F208V but not nudA F208I mutation causes a defect in dynein function.

To study the regulation of dynein-mediated retrograde transport of early endosomes, we used UV mutagenesis to obtain mutants that are defective in early endosome transport. The strain we used for mutagenesis contains the GFP-nudA allele that replaces the endogenous nudA and reports HC localization (57) and the mCherry-RabA allele that reports the distribution of early endosomes (45, 47). One mutant we selected was called 4-63 that formed a colony smaller than the wild type (Figure 1A). In this mutant, GFP-HC formed normal comet-like structures near the hyphal tip (Figure 1B, arrows), which represent dynein accumulation at the microtubule plus ends (41). However, a prominent buildup of mCherry-RabA-labeled early endosomes was observed in almost every hyphal tip, indicating a defect in dynein-mediated retrograde transport of early endosomes (Figure 1B, arrows). The mutant also exhibited an obvious defect in nuclear distribution (Figure 1C). Specifically, a cluster of multiple nuclei in the spore head was often observed (Figure 1C, arrows). This phenotype differs from that of the null mutant of p25, a dynactin subunit required for dynein-early-endosome interactions but not for nuclear positioning (59). Based on this phenotype, we concluded that the corresponding protein, unlike p25, is likely to be required for dynein activity rather than being specifically required for dynein’s physical interaction with early endosomes.

During an analysis of more than 50 progenies from a cross between the GFP-HC-containing mutant and a wild-type strain without GFP-HC, we noticed that the mutant phenotypes of colony growth and early endosome accumulation were always linked with each other and also with the presence of plus-end comet signals formed by GFP-HC. This suggests that the colony phenotype and the defect in early endosome transport were caused by the same mutation that is located within or very close to the nudA gene encoding dynein HC.

To identify the mutation, we first prepared five different wild-type genomic DNA fragments covering the entire 14-kb nudA gene by using high-fidelity PCR. Each nudA fragment was then co-transformed into the mutant with the auto-replicating pAid plasmid containing the selective marker gene pyrG (55). The 3-kb fragment containing sequences encoding the N-terminal part of HC was able to rescue the mutant phenotype via homologously replacing the equivalent sequence in the mutant genome. The corresponding region of the mutant was fully sequenced and one single mutation, T622G (in the coding sequence), was found when compared to the wild type nudA.
homologous recombination, and produce a colony fragment was able to rescue the mutant via essentially identical to wild type (Figure 2D). The nudA fragment containing the codon change of big region containing amino acids 300-1140 (60), dimerization site on the rat HC was mapped to a Dictyostelium discoideum fragments of cytoplasmic dynein HCs from rat and studies were done using in vitro expressed HC LIC interaction to the tail region of the HC. These of HC dimerization, HC-IC interaction and HC-HC tail. Previous in vitro studies mapped the sites upstream of the subunit interaction sites within the implicated in motor activity and it is also more hydrophobicity of the side chain may be responsible for the observed phenotype produced by the nudA F208V mutation. To test this, we mutated the F208 residue to isoleucine that also contains a branched side chain like valine but contains one extra –CH₃ group. We made a DNA fragment containing the codon change of nudA F208I, and introduced it to the nudA F208V mutant. This fragment was able to rescue the mutant via homologous recombination, and produce a colony essentially identical to wild type (Figure 2D). The nudA F208I mutant also exhibited normal nuclear distribution and early endosome distribution (Figure 2E, F). The striking phenotypic difference between the nudA F208V mutant and the nudA F208I mutant indicates that a subtle change in the side chain of F208 makes a significant difference in the function of dynein HC.

The nudA F208V mutation in the dynein tail does not affect dynein complex assembly, but affects the cargo-transporting capacity of dynein

The nudA F208V mutation locates at a site far upstream of the linker region before AAA1 implicated in motor activity and it is also more upstream of the subunit interaction sites within the HC tail. Previous in vitro studies mapped the sites of HC dimerization, HC-IC interaction and HC-LIC interaction to the tail region of the HC. These studies were done using in vitro expressed HC fragments of cytoplasmic dynein HCs from rat and Dictyostelium discoideum (60, 61). The HC dimerization site on the rat HC was mapped to a big region containing amino acids 300-1140 (60), which corresponds to a region containing amino acids 325-1180 of A. nidulans HC. However, the study using Dictyostelium HC fragments mapped the dimerization site to a region containing amino acids 627-780 (61), corresponding to amino acids 603-759 of A. nidulans HC. The IC binding site within the D. discoideum HC is in a region containing amino acids 629-730 (61), corresponding to amino acids 605-690 of the A. nidulans HC. However, the IC binding site of the rat HC contains amino acids 446-701 (60), corresponding to amino acids 470-720 of A. nidulans HC. Within the rat HC, the LIC binding site contains amino acids 649-800 (60), which correspond to amino acids 668-816 of A. nidulans HC. All of these sites are downstream of the nudA F208V mutation we identified.

Beside the positions of the deduced subunit binding sites in A. nidulans HC, several lines of experimental evidence also helped us to exclude the possibility that the nudA F208V mutation affects the HC-IC and/or HC-LIC interactions. First, while loss of IC or LIC in A. nidulans abolishes microtubule plus-end comets formed by the dynein HC (48, 50), GFP-NUDAF208V formed comet-like structures just like wild-type HCs (Figure 1B). Second, HC was pulled down with S-tagged IC from extract isolated from the nudA F208V mutant (Figure 3A). Similarly, the amount of IC associated with GFP-NUDAF208V was normal as judged by pull-down experiments using a GFP-antibody coupled to magnetic beads (Figure 3B), and the same conclusion was obtained even when the pull-down experiments were performed using high-salt buffers (Figure 3C). Since loss of dynein LIC is known to abolish HC-IC interaction (50), we concluded that neither HC-IC nor HC-LIC interaction is weakened by the nudA F208V mutation. This conclusion was further confirmed by a sucrose gradient sedimentation experiment showing normal sedimentation of the mutant dyenin complex (Figure 3D). Thus, we have identified a novel mutation in the dynein tail that clearly affects dynein function but not dynein complex assembly. In addition, our biochemical data also indicate that the nudA F208V mutation does not weaken the dynein-dynactin interaction (Figure 3C), which is expected since IC is the subunit that binds to dynactin (15-17), and since dynein forms normal plus-end comets (Figure 1B), which is dynactin dependent (49, 51-53).
Moreover, the nudA<sup>F208V</sup> mutation does not affect the interaction between dynein and the LIS1 homolog NUDF (Figure 3C).

To explore the mechanism of the nudA<sup>F208V</sup> mutation, we examined dynein-mediated movements of early endosomes in more detail in both the mutant and wild-type control strains. We first used a minimal growth medium containing glycerol as a carbon source, which allows the expression of the early endosome marker mCherry-RabA under the control of the alcA promoter. Under this condition, early endosomes were clearly blocked at the hyphal tip in the mutant as shown in Figure 1B. The frequency of early-endosome movement away from the hyphal tip in the mutant was about seven times lower than that in wild type. Because the number of early endosomes that move away from the hyphal tip was very low, it was not easy to do a quantitative analysis on the speed of movement in the mutant. We next used another minimal medium containing fructose and threonine, which allows a robust hyphal growth and a relatively low level of expression of the alcA promoter. In this medium, the signal intensity of mCherry-RabA in the wild type control was quite low compared to that in the glycerol-containing medium, most likely reflecting the relatively low expression level of the alcA-driven mCherry-RabA fusion protein. Under this condition, dynein-mediated movements of early endosomes were still blocked in the mutant, as evidenced by the abnormal accumulation of mCherry-RabA signals at the hyphal tip (Figure 4A, Supplemental Movies 1 and 2). However, more retrograde movements were observed, allowing for a quantitative analysis of the movement speed. Measurements of kymographs (Figure 4B) showed that while some early endosomes in the mutant moved as quickly as those in the wild type control, the frequency of the slower early endosomes was increased. Out of 53 early endosomes we measured from the mutant, 9 of them (~17%) moved at a speed below 1 µm/s, which was never observed in wild type (n=55) (Figure 4C). Consequently, the average speed of dynein-mediated movements in the mutant was mildly reduced (Figure 4D). The frequency of movements from the hyphal tip was about three times lower in the mutant than in the wild type (Figure 4E). Sometimes, early endosomes in the mutant underwent short-distance movements away from the tip but moved right back to the hyphal tip (Supplemental Movie 3). Under these conditions, we also observed a cloud of GFP-HC signals at the hyphal tip region where early endosomes also accumulated (Supplemental movies 4-6). This cloud was also observed in the mutant grown on glucose that shuts off the expression of mCherry-RabA, but not observed in the wild type strain grown under the same conditions (Supplemental movies 7 and 8). Because early endosomes also accumulate at the hyphal tip region, this localization pattern of dynein suggests that the mutant dynein is likely to interact with early endosomes. Such an idea is consistent with the result that the mutant HC is able to bind to IC, which binds dynactin (15-17), a complex required for dynein-early-endosome interaction (59, 62). To confirm this idea, we used a biochemical pull-down assay to directly examine the physical interaction between the dynein HC labeled with GFP and the early endosome cargo labeled with mCherry-RabA. The GFP-antibody-conjugated magnetic beads pulled down dynein-bound early endosomes from cell extract, which were detected on western blots probed by an anti-mCherry antibody (Figure 5A). A strain containing mCherry-RabA but not GFP-HC was used as a negative control for this assay, and the amount of mCherry-RabA pulled-down from the extract of this negative control strain was negligible (Figure 5A). The result of this assay showed that the mutant dynein is clearly able to bind early endosomes. In fact, the amount of mCherry-RabA pulled down from the mutant extract was even higher than that from the wild type extract (Figure 5A, <i>p</i>&lt;0.05, <i>n</i>=3), possibly because recycling of the mutant dynein from non-motile early endosomes is relatively inefficient. Together, our results suggest that rather than negatively affecting dynein-cargo interaction, the nudA<sup>F208V</sup> mutation affects the ability of cargo-bound dynein in initiating or sustaining movements along microtubules in vivo.

Two suppressor mutations of the nudA<sup>F208V</sup> mutant, nudA<sup>T1133S</sup> and nudA<sup>N356K</sup>, are also located in the HC tail.

To gain further insight into the structural role of the nudA<sup>F208</sup> residue, we performed a UV mutagenesis on the nudA<sup>F208V</sup> mutant. The spores
were mutagenized as described previously, and plated out on YUU plates. From about 8,000 colonies, two strains, RQ47 and RQ49, were selected. These two strains looked healthier than the original mutant on plates (Figure 6A), but less healthy than the wild type, indicating only a partial recovery of dynein function. As expected, the defects in dynein-mediated early endosome transport and nuclear distribution were only partially but not fully suppressed (Figure 6B-E). To test if the suppressor mutations were located in the \textit{nudA} gene, we crossed the selected suppressor with a wild-type strain. We reasoned that if the suppressor mutation is linked with \textit{nudA}^{F208V}, none of the progeny should look like the original mutant. This turned out to be the case for both suppressors. To identify the suppressor mutation in RQ47, we sequenced the entire \textit{nudA} gene from the selected suppressor mutant. Beside the original \textit{nudA}^{F208V} mutation as expected, we found only one additional mutation, \textit{nudA}^{T1133S}, which is also located in the dynein tail but downstream of the previously mapped subunit interaction sites. This Thr (T) residue is not highly conserved (Figure 6F), and how this subtle mutation (Thr to Ser) influences tail structure will need to be studied in the future. Similarly, the suppressor mutation \textit{nudA}^{F208V} in \textit{S. pombe} was found. This Asn (N) residue is conserved in most species examined but it is changed to Lys (K) in \textit{D. discoideum} HC and to Asp (D) in \textit{S. pombe} HC (Figure 6G). To verify that the improved colony phenotype of the original \textit{nudA}^{F208V} mutant was indeed caused by these mutations, we transformed the fragments containing either one of the suppressor mutations back to the original \textit{nudA}^{F208V} mutant. Both fragments generated transformants that looked like the originally selected suppressors, and sequencing analyses of two of these suppressor-like transformants confirmed the presence of the two additional mutations. Thus, the \textit{nudA}^{T1133S} or the \textit{nudA}^{N356K} mutation partially compensates for the structural alteration caused by the \textit{nudA}^{F208V} mutation.

**Discussion**

In this study, we have identified a novel mutation, \textit{nudA}^{F208V} in the \textit{A. nidulans} dynein HC tail that significantly weakens dynein function in nuclear migration and early endosome movement. Although the dynein tail has been implicated in dynein subunit interactions, this mutation is located upstream of the subunit interaction sites and does not apparently affect dynein complex assembly. In the \textit{nudA}^{F208V} mutant, early endosomes abnormally accumulate at the hyphal tip, which is indicative of a defect in dynein-mediated early endosome movement. The physical interaction between the dynein motor and the early endosome cargo is clearly not weakened. However, the frequency of dynein-mediated early endosome movement away from the hyphal tip is significantly reduced. Moreover, while some early endosomes that leave the hyphal tip move at a normal speed, some move at an abnormally low speed. Together, these results suggest that the \textit{nudA}^{F208V} mutation causes a defect of dynein in initiating and/or sustaining cargo movement along the microtubule track in vivo.
the same cargo. In this scenario, dynein is likely to be detached from the microtubule or take backward steps as suggested by an in vitro study examining yeast dynein under a load that is bigger than its stall force (69). The abnormal accumulation at the hyphal tip in the nudA^{F208V} mutant clearly suggests a weakened ability of the mutant dynein to compete against kinesin-3. This may be resulted from a decreased microtubule affinity during the ATPase cycle causing the dynein motor to detach from the track, or a decreased stall force causing the motor to reverse direction more frequently. Future in vitro work applying the optic trap technique will need to be done to address these possibilities.

While several reports have described the structure of the HC motor domain (70-76), the structure of the tail domain has not yet been reported. Thus, how the nudA^{F208V} mutation affects tail structure remains unknown. However, identification of the nudA^{F208V} mutation and its suppressor mutations in the dynein tail indicates that the fine structure of the tail is important for motor function in vivo. It is interesting to note that the nudA^{F208I} mutation does not cause any obvious defect, suggesting that the proper size or hydrophobicity of the side chain in the F208 residue is critical for function. Recently, a genetic study in another filamentous fungus Neurospora crassa has identified many mutations in the dynein HC that affect dynein function (77). While most mutations are in the motor domain, two mutations, Y110S and W1308G, of the N. crassa HC tail have been identified (77). Interestingly, like the the nudA^{F208V} mutation, the Y110S mutation is also located far upstream of the subunit interaction site. Further insights into structural roles of these involved amino acids will be generated after the structure of the tail becomes available. Exactly how a tail mutation would affect dynein motor function is unclear at this point. It has been postulated that the budding yeast dynein tail interacts with the motor domain in a way that the motor domain would “mask” the tail (78, 79). Interestingly, while the dynein regulator LIS1 binds to the motor domain at AAA3/AAA4 (26), removal of the tail appears to enhance dynein-LIS1 interaction (31, 78). Given that the tail is involved in multiple protein interactions (14-18, 21-25), it remains to be tested whether the nudA^{F208V} mutation affects dynein function directly through regulating the motor domain or indirectly through coordinating the functions of other dynein regulators.

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**Figure 1** Phenotypes of the 4-63 (later identified as \textit{nudA}^{F208V}) mutant. (A) The mutant forms a colony that is smaller than wild type. (B) The mutant exhibits normal dynein localization to microtubule plus ends as evidenced by the presence of comet-like structures near the hyphal tip (indicated by arrows), but it shows a severe defect in the retrograde movement of early endosomes as evidenced by an abnormal accumulation of the mCherry-RabA signals at the hyphal tip (the hyphal tips in the wild type and the mutant are indicated by arrows). (C) The mutant exhibits an obvious defect in nuclear distribution as evidenced by DAPI staining showing a cluster of multiple nuclei in the spore head (the spore heads in the wild type and the mutant are indicated by arrows). Bar, 5 \( \mu \text{m} \).
Figure 2 Identification of the \( nudA^{F208V} \) mutation as the 4-63 mutation in dynein HC. (A) Sequence of the mutant genomic DNA showing the T622G mutation in the coding region of \( nudA \). This change is predicted to cause the \( nudA^{F208V} \) mutation in the HC tail. (B) A diagram showing the position of the mutation in the HC tail (red star). The original diagram was published in Schmidt et al. (2012) (74). (C) A sequence alignment showing that the F208 residue is conserved in some species but changed to other amino acids such as isoleucine and leucine in the dynein HCs of other organisms. (D) The \( nudA^{F208I} \) mutant forms a wild-type colony. (E) The \( nudA^{F208I} \) mutant exhibits normal nuclear distribution with no cluster of multiple nuclei at the spore head. The spore heads are indicated by arrows. (F) The \( nudA^{F208I} \) mutant exhibits normal early endosome distribution with no buildup of early endosomes at the hyphal tip. The hyphal tips are indicated by arrows. Bar, 5 \( \mu \)m.
Figure 3 The nudA<sup>F208V</sup> mutation does not weaken the HC-IC interaction. (A) The amount of the nudA<sup>F208V</sup> mutant HC pulled down with the S-tagged IC (S-IC) is normal compared with that of the wild type HC. The lane labeled as “negative control” contains the sample from a wild type strain whose IC is not tagged with the S-tag. (B) The amount of IC pulled down by the GFP-antibody from the extract containing GFP-nudA<sup>F208V</sup> is normal compared to that from the extract containing GFP-nudA. The lane labeled as “negative control” contains the sample from a wild type strain whose HC is not tagged with GFP. The experiment was done using protein extraction buffer containing detergent but no NaCl. (C) The amount of dynein IC, p150 of dynactin and NUDF/LIS1 pulled down by the GFP-antibody from the extract containing GFP-nudA<sup>F208V</sup> is similar to that from the extract containing GFP-nudA. The experiment was done using protein extraction buffer containing 0 mM, 50 mM or 100 mM NaCl. (D) The mutant HC is incorporated into the dynein complex as indicated by its normal sedimentation at around 19S in a 5-25% sucrose gradient. The experiment was done using a protein extraction buffer containing 50 mM NaCl. Thymoglobulin (Sigma) was used as a 19S marker.
Figure 4 A quantitative analysis of dynein-mediated retrograde transport of early endosomes in the nudA^{F208V} mutant. (A) Images showing distribution patterns of early endosomes in the wild type and the nudA^{F208V} mutant strains. Positions of the hyphal tips are indicated by arrows. Bar, 5 µm. (B) Kymographs from the same time-lapse images of the wild type and mutant cells shown in A. Arrows point to the lines that represent retrograde transport from the hyphal tip. The kymograph generated from the mutant shows an early endosome that first left the hyphal tip and then came back. (C) An analysis of the speed distribution of retrograde early endosome movement away from the hyphal tip. The percentage of early endosomes that move at various speeds is shown as the frequency value (n=55 early endosomes for the wild type, and n=53 for the mutant). (D) Mean and S.D. values of the speed of dynein-mediated early endosome movement away from the hyphal tip. The mean speed value of the mutant is mildly reduced (p<0.05; n=55 for the wild type, and n=53 for the mutant). (E) An analysis of the frequency of early endosome movements away from the hyphal tip. Mean and S.D. values are shown (n=68 hyphal tips for the wild type and n=64 for the mutant). The average frequency of movements in the mutant within a time period of 16 seconds is relative to that of the wild type, which is set as 1. The frequency is significantly lower in the mutant than in wild type (p<0.001).
Figure 5 A biochemical analysis showing that dynein in the \textit{nudA}^{F208V} mutant is able to interact with mCherry-RabA-labeled early endosomes. (A) Western blots showing a result of the pull-down assay for dynein-early-endosome interaction using an anti-GFP antibody conjugated to magnetic beads. The amount of mCherry-RabA pulled down with the GFP-HC from the \textit{nudA}^{F208V} mutant extract is clearly not lower than that from the wild type extract. The lane labeled as “negative control” contains the pull-down sample from a strain containing mCherry-RabA but not GFP-HC. (B) A quantitative analysis of the results of the pull-down assays for dynein-early-endosome interaction. The intensity ratio of the mCherry-RabA band to that of the GFP-HC band is shown. Values are relative to the wild-type value, which is set at 1. Mean and S.D. values were calculated from three independent pull-down experiments.
Figure 6 Analyses of the two suppressors of the nudA<sup>F208V</sup> mutant. (A) Both the nudA<sup>N356K</sup> and nudA<sup>T1133S</sup> mutations partially suppress the colony phenotype of the nudA<sup>F208V</sup> mutant. (B) Nuclear-distribution phenotypes of the suppressors. Percentage of cells in which the spore head contains four or more nuclei is presented. The mean and S.D. values are from data of three independent experiments (n>200 DAPI-stained cells for each strain). The mean value of the nudA<sup>F208V,N356K</sup> or nudA<sup>F208V,T1133S</sup> mutant is lower than that of the nudA<sup>F208V</sup> mutant (p<0.001). (D) Early-endosome-distribution phenotypes of the suppressors. (E) A quantitative analysis on the frequency of dynein-mediated early endosome movements. Mean and S.D. values are shown (n=35 hyphal tips for wild type, n=45 for the nudA<sup>F208V</sup> mutant, n=49 for the nudA<sup>F208V,N356K</sup> mutant and n=49 for the nudA<sup>F208V,T1133S</sup> mutant). The mean values of frequencies in the mutants within a time period of 16 seconds are relative to that of the wild type, which is set as 1. The mean value of the nudA<sup>F208V,N356K</sup> or nudA<sup>F208V,T1133S</sup> mutant is higher than that of the nudA<sup>F208V</sup> mutant (p<0.001). (F) A sequence alignment of dynein HCs showing the position of the suppressor mutation at T1133. (G) A sequence alignment of dynein HCs showing the position of the suppressor mutation at N356.
Table 1 *A. nidulans* strains used in this study

| Strain name | Genotype | Source |
|-------------|----------|--------|
| GR5         | *pyrG89; pyroA4; wA3* | G. S. May |
| TNO2A3      | Δ*nkuA::argB; pyrG89; pyroA4* | (80) |
| JZ11 or S-IC | S-tagged-*nudI; pyrG89; pabaA1, yA1* | (57) |
| LZ12        | GFP-*nudA; nkuA::argB; pyroA4; pyrG89* | (57) |
| LZ26        | GFP-*nudA; S-tagged-*nudI* (or S-IC); nkuA::argB; pyroA4; pyrG89, yA1 | (57) |
| XX222       | GFP-*nudA; argB2::*argB*-alcAp::mCherry-RabA*; pantoB100 | (47) |
| JZ476       | GFP-*nudA*F208V*; S-IC; wA2; possibly *pyroA4*; possibly *pabaA1*; possibly *yA1* | This work |
| RQ2         | GFP-*nudA; argB2::*argB*-alcAp::mCherry-RabA*; nkuA::argB; pyrG89; pyroA4; yA1 | This work |
| RQ5         | GFP-*nudA*F208V*; argB2::*argB*-alcAp::mCherry-RabA*; pyrG89; pyroA4; wA2 | This work |
| RQ8         | GFP-*nudA*F208V*; argB2::*argB*-alcAp::mCherry-RabA*; pyrG89; wA2 | This work |
| RQ16        | GFP-*nudA; argB2::*argB*-alcAp::mCherry-RabA*; nkuA::argB; pyrG89; wA2 (with pAid) | This work |
| RQ39        | GFP-*nudA*F208I*; argB2::*argB*-alcAp::mCherry-RabA*; pyrG89; wA2 (with pAid) | This work |
| RQ47        | GFP-*nudA*F208V*T1133S*; argB2::*argB*-alcAp::mCherry-RabA*; pyrG89; wA2 | This work |
| RQ49        | GFP-*nudA*F208V*N356K*; argB2::*argB*-alcAp::mCherry-RabA*; pyrG89; wA2 | This work |
| RQ54        | argB2::*argB*-alcAp::mCherry-RabA*; nkuA::argB; pyrG89; pyroA4; wA2 | This work |
Identification of a novel site in the tail of dynein heavy chain important for dynein function in vivo
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