Point mutations in the stem region and the fourth AAA domain of cytoplasmic dynein heavy chain partially suppress the phenotype of NUDF/LIS1 loss in *Aspergillus nidulans*

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Abstract:

Cytoplasmic dynein performs multiple cellular tasks but its regulation remains unclear. The dynein heavy chain has a N-terminal stem that binds to other subunits and a C-terminal motor unit that contains six AAA (ATPase associated with cellular activities) domains and a microtubule-binding site located between AAA4 and AAA5. In *Aspergillus nidulans*, NUDF (a LIS1 homolog) functions in the dynein pathway, and two *nudF* partial suppressors were mapped to the *nudA* dynein heavy chain locus. Here we identified these two mutations. The *nudAL1098F* mutation resides in the stem region, and *nudAR3086C* is in the end of AAA4. These mutations partially suppress the phenotype of *nudF* deletion but do not suppress the phenotype exhibited by mutants of dynein intermediate chain and Arp1. Surprisingly, the stronger Δ*nudF* suppressor, *nudAR3086C*, causes an obvious decrease in the basal level of dynein’s ATPase activity and an increase in dynein’s distribution along microtubules. Thus, suppression of the Δ*nudF* phenotype may be resulted from mechanisms other than simply enhancing dynein’s ATPase activity. The fact that a mutation in the end of AAA4 negatively regulates dynein’s ATPase activity but partially compensates for NUDF loss indicates the importance of the AAA4 domain in dynein regulation in vivo.
Introduction:

Cytoplasmic dynein is a microtubule motor that plays multiple roles in mitosis, organelle distribution, and transport of vesicles, proteins/mRNAs and viruses (reviewed by Vale 2003; Levy and Holzbaur 2006; Greber and Way 2006; Vallee and Hook 2006). However, it is not yet clear how cytoplasmic dynein is targeted to various cellular locations and how its motor activity is regulated. Cytoplasmic dynein in higher eukaryotic organisms has been purified as a multi-subunit complex with a molecular mass greater than 1 mDa. It consists of heavy chains (~500 kDa), intermediate chains (~74 kDa), light intermediate chains (50-60 kDa) and light chains (8 kDa, 14kDa and 22kDa) (reviewed by Pfister et al., 2006). Many proteins involved in cytoplasmic dynein function have been discovered, including proteins in its accessory complex, dynactin (reviewed by Schroer 2004), and also the LIS1 protein (reviewed by Gupta et al., 2002; Hatten 2005). Lis1 was initially identified as a causal gene for human lissencephaly, a disease associated with abnormal brain development (Reiner et al., 1993). The connection between LIS1 homologs and cytoplasmic dynein was first made in the filamentous fungus *Aspergillus nidulans* and the budding yeast *Saccharomyces cerevisiae* by genetic studies (Xiang et al., 1995; Geiser et al., 1997). Further studies have demonstrated that LIS1 and its homologs in higher eukaryotic systems are also involved in cytoplasmic dynein function, and physical interactions between LIS1 and dynein/dynactin have been shown (Liu et al., 2000; Dawe et al., 2001; Sasaki et al., 2000; Tai et al., 2002; reviewed by Wynshaw-Boris and Gambello, 2001; Gupta et al., 2002; Tsai and Gleeson, 2005; Vallee and Tsai, 2006). Recently, purified LIS1 has been shown to enhance the microtubule-stimulated ATPase activity of the dynein motor (Mesngon et al., 2006).
Cytoplasmic dynein heavy chain, which contains the ATPase and the microtubule-binding domains, is responsible for motility. The N-terminal one third of the protein forms the stem region, which includes the sites for heavy chain dimerization, and interaction sites between heavy chain and intermediate chain, light intermediate chain and LIS1 (Habura et al., 1999; Tynan et al., 2000; Tai et al., 2002). The stem region is followed by the motor head containing six AAA domains that form a ring-like structure (Samso et al., 1998; Burgess et al., 2003). AAA1 has been shown to be the major ATP hydrolysis site based on the UV-vanadate-mediated photocleavage assay (Gibbons et al., 1987). But mutational analyses suggest that ATP binding and hydrolysis at AAA3 are also critical for dynein function and allow dynein to be released from microtubules (Silvanovich et al., 2003; Reck-Peterson and Vale 2004; Kon et al., 2004). ATP hydrolysis at AAA2 or AAA4 does not seem to be essential (Reck-Peterson and Vale 2004), but ATP binding at AAA2 or AAA4 may enhance the microtubule-binding and the ATPase activity of dynein (Reck-Peterson and Vale 2004; Kon et al., 2004). Between the fourth and the fifth AAA domains, there is a microtubule-binding stalk of about 10-15 nm in length (Goodenough and Heuser 1984; Gee et al., 1997; Samso et al., 1998; Burgess et al., 2003). It is not entirely clear how these domains affect each other during the ATPase cycle to produce mechanical force. Electron microscopic analyses of a flagella dynein have suggested that the stem connects to the motor head through a linker of about 10 nm long, and a change in the orientation of the linker correlates with dynein power stroke that results in an about 15 nm displacement of the tip of the microtubule binding stalk (Burgess et al., 2003). This linker may correspond to a region, about 600 amino acids before the first AAA domain, which may affect the ATPase cycle of the
AAA1 domain (Gee et al., 1997; Vallee and Hook 2006). Taken together, the dynein motor is organized in a unique fashion different from other motors such as kinesins and myosins (King 2000; Asai and Koonce 2001; Burgess and Knight 2004; Koonce and Samso 2004; Vallee and Hook 2006). Not only the structure of the dynein motor is complex, recent studies on dynein behaviors have added another layer of complexity onto this motor (Mallik et al., 2004; Ross et al., 2006; Toba et al., 2006; Reck-Peterson et al., 2006). For example, unlike conventional kinesins that walks in one direction towards the plus end, the cytoplasmic dynein-dynactin complex exhibits energy-dependent bidirectional movements in single molecule motility assays (Ross et al., 2006).

In live cells, proteins in cytoplasmic dynein and/or dynactin complex form comet-like structures representing their accumulation at the microtubule plus end, a site implicated in microtubule-cortex interaction and in dynein cargo loading (Han et al., 2001; Vaughan et al., 2002; Lee et al., 2003; Sheeman et al., 2003; Lenz et al., 2006). In A. nidulans, the plus-end accumulation of cytoplasmic dynein depends on a conventional kinesin-KINA and dynactin, but deletion of NUDF (LIS1 homolog) makes the comets longer, thereby increasing the sum of comet intensity (Zhang et al., 2003). These results are very similar to that from a dimorphic fungus Ustilago maydis in which the microtubule plus end localization of dynein is implicated in transporting endosomes from the microtubule plus end towards the minus end in hyphae (Lenz et al., 2006). Consistently, dynein comets are more prominent in the absence of NUDF-interacting protein NUDE/RO11 in A. nidulans and Neurospora crassa (Minke et al., 1999; Efimov 2003). Thus, while LIS1 homologs in filamentous fungi may be targeted to the microtubule plus end via mechanisms different
from that of dynein’s plus-end targeting (Efimov et al., 2006), they are not required for dynein’s plus-end localization, but instead, they may facilitate dynein’s departure from the microtubule plus end. It is important to note, however, that in *S. cerevisiae*, the LIS1 homolog Pac1p is required for dynein’s microtubule-plus-end accumulation (Lee et al., 2003; Sheeman et al., 2003). These results suggest that LIS1 homologs may regulate dynein in multiple modes.

In a previous genetic study in *A. nidulans*, Willins et al. (1997) isolated extragenic suppressors of the nudF6 mutation (*snf*) that partially suppress the nudF6 phenotype, and two of the mutations in the *snf/C* locus were shown to locate within or extremely close to the dynein heavy chain gene (Willins et al., 1997). To gain better insights from these initial findings, we have now sequenced the heavy chain genes of the suppressor strains and found the positions of these mutations. These two mutations are located in two different domains of the dynein heavy chain: one, *nudA*L1098F, is in the stem region; and the other, *nudAR3086C*, is in the end of the fourth AAA domain (AAA4), but not in the ATP-binding (Walker A) or the hydrolysis (Walker B) site. These mutations partially suppress the ∆nudF mutant phenotype but not the phenotype of dynein intermediate chain and Arp1 mutants. The *nudAR3086C* mutation located in the end of AAA4 is a relatively stronger suppressor of nudF mutants. Interestingly, while dynein containing this mutation interacts with NUDF just like wild type dynein, its basal level ATPase activity is obviously decreased. This is also correlated with an increase in dynein’s distribution along cytoplasmic microtubules. We suggest that these mutations may specifically alter dynein to partially compensate for NUDF loss, but the alterations may not necessarily increase dynein’s ATPase activity.
Materials and Methods:

1. Strains and Aspergillus techniques.

*A. nidulans* strains are listed in Table 1. Media, growth conditions, genetic crosses, *A. nidulans* transformations and genomic DNA isolation were as described previously (Xiang *et al.*, 1995; Willins *et al.*, 1995; 1997). In many experiments described in this paper, we transformed *A. nidulans* strains in which the *A. nidulans* Ku70 homolog was deleted (*Δnkua*) (Nayak *et al.*, 2006), with individual genomic fragments plus the plasmid pAid containing the *pyrG* selective marker (Xiang *et al.*, 1999). The *Δnkua* strains made most of the described experiments feasible due to an increased frequency of homologous integration of a genomic fragment into the *A. nidulans* genome (Nayak *et al.*, 2006).


Overlapping regions of the *nudA* genomic DNA were amplified with multiple sets of primers in the *nudA* coding sequence. The PCR products were sequenced and the sequence data were analyzed using the DNA Star program. Oligoanes used for PCR amplification of the *nudA* genomic DNA and for sequencing analyses are listed in Supplemental Table 1.

3. Construction of a GFP-*nudA* strain in which the GFP-*nudA* fusion is under the control of its own promoter.

We have previously constructed a strain in which GFP is inserted in the *nudA* coding region between the 8th and the 9th aa, and the GFP-*nudA* fusion is under the control of the
alcA promoter (Xiang et al., 2000). On medium containing glucose, the expression of the GFP-nudA fusion is repressed, and our previous observations on GFP-NUDA were all done using medium containing glycerol as the carbon source. In this study, we constructed a new strain in which the GFP-nudA fusion is under the control of the endogenous promoter of nudA, and the GFP-NUDA fusion protein in such a strain can be observed using regular medium containing glucose. The following method was used to make this new strain. A 1.5 kb fragment upstream of the nudA coding sequence was amplified from genomic DNA of the wild type strain GR5 by using the following primers: NudAupper (5’-AGTAAGCCAAGTTTCTACCGA-3’) and NudAlower (5’-TTGCGGCGATAGAGTTTTCCCGCGACGCTGGTCCGTAAC-3’). A 2.3 kb SmaI and BamHI fragment was obtained from the original alcA-GFP-nudA plasmid (Xiang et al., 2000). Subsequently, a fusion PCR was performed to fuse the 1.5 kb and the 2.3 kb fragments with the following primers: NudAupper (5’-AGTAAGCCAAGTTTCTACCGA-3’) and NudAend (5’-CTTCATTAAGGTGCAGGAATTCGCG-3’). This resulted in a 3.8 kb product that contains the nudA upstream region followed by the nudA coding sequence with GFP inserted. This fragment was co-transformed with the auto-replicating plasmid pAid that contains the selective marker pyrG (Xiang et al., 1999) to the TNO2A3 strain with ΔnkuA (Nayak et al., 2006). Transformants were screened under a fluorescence microscope for the presence of the comet-like structures near the hyphal tip, which represent GFP-NUDA accumulated at the dynamic microtubule plus ends. Several strains with positive GFP signals were selected. A strain (LZ12) that subsequently lost the auto-replicating
plasmid pAid after growing on non-selective medium Y+UU plus FOA, was used for further experiments.

The GFP-\textit{nudA} strain was crossed to \textit{nudF} mutant strains, and the strains carrying both GFP-\textit{nudA} and the \textit{nudF} mutations were obtained. To screen these strains for the presence of Δ\textit{nkuA}, we sequenced the \textit{nKuA} locus using primers ktF: 5'-cgtcgtacaggtaccaggactttc-3' and ktR: 5'-ctgcaattattgcatgcgtttc-3' (Nayak et al., 2006). The Δ\textit{nkuA}-positive strains were then transformed with fragments to confirm that the identified \textit{nudA} dynein heavy chain gene mutations cause suppression of the \textit{nudF} mutant phenotype.


We constructed a plasmid containing a N-terminal truncated \textit{nudI} (dynein intermediate chain) gene tagged at its C-terminal coding region with an S-tag (a peptide in RNase A). Specifically, a DNA sequence encoding a 15- amino acid S-tag peptide (Novagen) was added to the 3’-end of \textit{nudI} coding region right before the stop codon. The \textit{nudI} coding region and its 3’-untranslated region were used as templates in PCR reactions using the following primer sets: 5’-AAA GAA ACC GCT GCT GCT AAA TTC GAA CGC CAG CAC ATG GAC AGC TAA TTG ATT GGA TCT AC-3’ (S-tag1) and 5’- CCC CAC CGC GGT GGC AAT AAT GAA TAG GGA C 3’ (zh5), 5’-GCT GTC CAT GTG CGC TTC GAA TTT AGC AGC AGC GGT TTC TTT GCT CAT TCG GTC CTT ATC C-3’ (S-tag2) and 5’-AAG CGG CCG CAG ATG GCC TTT CAA G-3’ (zh1) (the
nucleotides corresponding to the S-tag are underlined). S-tag1 and zh5 were used to generate a PCR product containing the S-tag, the stop codon and nudI sequence downstream of the stop codon. S-tag2 and zh1 were used to generate a PCR product containing a 5’ truncated nudI coding region and the S-tag. The two PCR products were annealed and used as a template in a second PCR reaction with zh1 and zh5 as primers. The fusion PCR product was cloned into the NotI-BstXI sites of a vector pXX1 (containing the selective marker pyrG) (Xiang et al., 1995b) to generate a plasmid named “pS-tag”. The in-frame insertion of the S-tag right before the stop codon was confirmed by DNA sequencing. This plasmid was transformed into the nudI416 ts mutant to determine if S-tagged-nudI is functional. Ts+ transformants were obtained, indicating that pS-tag integrated into the nudI locus and rescued the nudI416 phenotype. The presence of the S-tag in the genome was confirmed by PCR reactions using chromosomal DNA as a template and with the primers: 5’ - GCTGGCGTTCGAATTTAGC - 3’ (S-tag3) and zh1. Using FOA selection, we obtained a strain in which the pyrG selective marker was lost, and this strain, JZ11 or S-IC, was used for genetic crosses to obtain strains with the suppressor mutations in the background of S-tagged-nudI.

5. A. nidulans dynein purification and ATPase assay.

A. nidulans protein extract was obtained from an overnight culture of 1-Liter using the liquid nitrogen grinding method for breaking the hyphae, which was similar to what has been described previously (Zhang et al., 2002), except that the protein isolation buffer contains 25 mM Tris (PH 8.0), 0.4% Triton X-100, 10 µM ATP, 1 mM DTT and a protease inhibitor cocktail (Sigma). The S-tag is a 15 amino acid peptide that binds to the
S-protein (the larger fragment of RNase A produced by subtilisin digestion) attached to agarose beads (Novagen, Inc.), and the S-tag-based purification method has been previously used to purify NUDF in *A. nidulans* (Ann and Morris 2000). For purification of *A. nidulans* dynein from strains with the S-IC background, about 30 ml of a protein extract (about 10 mg/ml) was incubated for half an hour at room temperature with 0.5 ml of S-protein beads (Novagen, Inc.). The beads were repeatedly washed with the same buffer used for protein isolation except that no detergent was added. Finally, the S-tagged protein was eluted with 5 mg/ml S-peptide in 0.2 ml of a buffer containing 25 mM Tris HCl (pH 8.0), 10 µM ATP and 1 mM DTT. About 30 µl of the eluate was loaded onto a 4-15% SDS-PAGE gradient gel (Bio-Rad). Dynein heavy chain, intermediate chain, and NUDF were detected by Western blot analyses using an antibody against NUDA (Xiang *et al*., 1995b), S-protein-conjugated alkaline phosphatase (Novagen, Inc.) and an anti-NUDF antibody (Xiang *et al*., 1995a). For silver staining of protein gels, the Silver Stain Plus kit from Bio-Rad was used.

ATPase assays were done by incubating a mixture of 30 µl containing the S-peptide-eluted fraction, 25 mM Tris HCl (pH 8.0), 10 mM MgCl₂ and 1 mM ATP at 37°C for 1 hour. 20 µl of the mixture were measured for the level of free phosphate using the PhosFree Phosphate Assay Biochem Kit (Cytoskeleton, Inc.). A multiple-channel pipette was used to simultaneously add reagents to eight individual samples contained in separate wells of a 96-well plate. The MRX Microplate Reader with the Revelation Software (Version 4.06) (Dynex Technologies, Inc.) was used for reading the O. D. values at 630 nm. Concentrations of the dynein heavy chain in different samples were estimated by
comparing the intensity of the heavy chain band to differently diluted BSA samples on the same silver-stained gel. The image of the gel was acquired using LAS-1000 (Fujifilm) with the 1R LAS-1000 Life software, and quantification of the bands was done using the Image Gauge software (version 4.01).

6. Microscope techniques.

Fluorescence microscopy of live *A. nidulans* hyphae was as described (Li *et al.*, 2005). Images were captured using an Olympus IX70 inverted fluorescence microscope (with a 100X objective) linked to a cooled CCD camera. The IPLab software (BioVision Technologies) was used for image acquisition and analysis. Cells were incubated at 32°C in ΔTC3 culture dishes (Bioptechs, Butler, PA) for overnight before observation at 32°C. Liquid minimal medium containing glucose and supplements was used. A Biotechs heating stage and heating objective system was used. Single GFP images were obtained by using a 0.1-second exposure time. For imaging cells with both CFP-*tubA* and GFP-*nudA*, Ludl Electronic Products dual individual excitation and emission motorized filter wheels were used. Chroma 8600 filters for CFP (430 nm peak excitation with a bandwidth of 25 nm, 470 nm peak emission with a bandwidth of 30 nm) were used for observing CFP-microtubule, and filters for YFP (500 nm peak excitation with a bandwidth of 20 nm, 535 nm peak emission with a bandwidth of 30 nm) were used for observing GFP-NUDA. A 0.2-second and a 0.6-second exposure times were used for capturing CFP-microtubule and GFP-NUDA images respectively.
Results:

1. The two snfC mutations reside in the N-terminal stem region and the end of the fourth AAA domain respectively.

We sequenced the entire coding region of the *A. nidulans* cytoplasmic dynein heavy chain gene (*nudA*) in the two snfC strains. We found that each snfC mutant contains one single base pair mutation. The *nudA* gene in the snfC1524 mutant contains a change in nucleotide #3294 (starting from ATG) from A to T, corresponding to a change in amino acid #1098 from Leucine (L) to Phenyalanine (F). This change is located in the dynein heavy chain’s stem region (Figure 1A). Interestingly, the snfC1232 mutation is located at the end of the fourth AAA domain, near the microtubule-binding stalk that contains coil-coiled motifs, which is far away from the snfC1524 mutation in the primary sequence (Figure 1A). The snfC1232 mutation changes nucleotide #9258 from C to T, and the corresponding amino acid #3086 is changed from Arginine (R) to Cysteine (C). We did a sequence alignment with dynein heavy chains from several species, and found that both L1098 and R3086 in the *A. nidulans* cytoplasmic dynein heavy chain are conserved from fungi to human, suggesting that they are important residues for dynein function (Figure 1B).

Because we only sequenced the coding regions of *nudA* in these two snfC strains, we were concerned about the possibility that other mutations upstream or downstream of the *nudA* coding sequence could be causally related to the partial suppression. To verify that the single mutations we found are the only ones that cause the partial suppression, we did
the following experiments. We first amplified two fragments within the *nudA* coding region and in each of them, the single mutation is located near the middle. The following two sets of primers: (1) *NudA*52 (5'-CAACTTCTGGTTATCCATGGA-3') and *NudA*36 (5'-TTGGATCTACCAGCATAGCCA-3'), and (2) *NudA*57 (5'-TCCAAGCTATGGGTCGTATCT-3') and *NudA*312 (5'-AGAGCATCGACCTTTAGCTT-3'), were used for PCR reactions to amplify a 5.2 kb *nudA* fragment containing the L1098F mutation and a 6.2 kb fragment containing the R3086C change respectively. Since the previous genetic study suggested that these mutations represent bypass suppressors (Willins *et al.*, 1997), we used different *nudF* alleles to test for suppression. To verify that the L1098F mutation in the stem region caused *nudF* suppression, the 5.2 kb fragment was transformed into a strain (LZ35) that carries the *nudF*7 mutation and the GFP-tagged *nudA* dynein heavy chain controlled by its own promoter. A transformant that formed a bigger, *nudF*7-suppressor-like colony was selected for sequencing analysis, and a codon change corresponding to the L1098F mutation was found. To rule out the possibility that other mutations in the 5.2 kb fragment introduced during the PCR procedure may contribute to the partial suppression, we sequenced the entire corresponding 5.2 kb genomic sequence of this transformant and confirmed that the L1098F change was the only amino acid change in this region. This result is consistent with the idea that the L1098F mutation partially suppressed the *nudF*7 mutant phenotype. To further demonstrate that the observed suppression is indeed linked to the mutation in *nudA*, we crossed this transformant to the Δ*nudF* mutant and analyzed the progeny. Because the *nudF*7 mutant only forms nud-like colonies at 42°C but the Δ*nudF* mutant forms nud-like colonies at any temperature including 32°C, we were able
to select the $\Delta nuf$ colonies and $\Delta nuf$-suppressor-like colonies at 32°C. If the suppressor mutation introduced into the $nuf$7 strain (with GFP-NUDA in the same genome) is linked to $nufA$, then the $\Delta nuf$-suppressor-like phenotype should be linked to the GFP-NUDA signal. Indeed, five randomly picked progeny with $\Delta nuf$-suppressor-like phenotype all showed GFP-NUDA signal under the microscope. These results strongly suggested that the L1098F mutation caused $nuf$ partial suppression phenotype observed in the $snf$$C$1524 mutant, and confirmed that the L1098F change represents a bypass suppressor rather than an allele-specific suppressor of $nuf$ (Figure 2).

To verify that the R3086C change caused the partial suppression phenotype observed in the original $snf$$C$1232/$nuf$6 strain, we used a slightly different strategy. Because $snf$$C$1232 by itself exhibited a conidiation phenotype (Figure 2) (Willins et al., 1997), we transformed the 6.2 fragment with the R3086C mutation to a wild type strain containing GFP-tagged $nufA$ under the control of its own promoter. A transformant that grew like a $snf$$C$1232 single mutant was selected and sequence analysis of the entire corresponding 6.2 kb region in the genomic DNA confirmed the presence of only the R3086C amino acid change. This strain was then crossed to the $\Delta nuf$ mutant, and both $\Delta nuf$ and $\Delta nuf$-suppressor-like colonies were analyzed. None of the five randomly selected $\Delta nuf$-like progeny had GFP signal while five suppressor-like progeny all had GFP-NUDA signal. Because the GFP-NUDA signal is linked to the R3086C change in the original transformant, our result strongly supports the conclusion that the R3086C change in the end of the fourth AAA domain partially suppresses the phenotype caused by loss of $nuf$. 
2. The *nudAR3086C* mutation acts as a stronger \(\Delta nudF\) suppressor than *nudAL1098F* and exhibits a mild growth defect.

The previous genetic study has suggested that both *snfC* alleles act as *nudF* bypass suppressors, but the extent of suppression was not shown (Willins *et al*., 1997). In this study, by using sequencing analyses and/or the linkage to the GFP-NUDA signal, we isolated strains containing each suppressor mutation in the \(\Delta nudF\) background.

Compared to *nudAL1098F*, *nudAR3086C* acts as a stronger suppressor of the \(\Delta nudF\) phenotype both at 32°C and at 42°C (Figure 2). The level of suppression by *nudAR3086C* on the *nudF*6 phenotype is also clearly higher than that by *nudAL1098F* (Figure 2), which is consistent with data from the previous study (Willins *et al*., 1997). It has been shown previously that *nudF* mutants exhibited a conidiation (asexual spore formation) defect (Xiang *et al*., 1995), and the suppressor mutations did not obviously restore conidiation of the *nudF* mutants. DAPI staining showed that the nuclear distribution defect in the vegetative hyphae of the \(\Delta nudF\) mutant was clearly suppressed partially by the *nudAR3086C* mutation, but not obviously suppressed by the *nudAL1098F* mutation (Figure 3). The *nudAR3086C* mutation also acted as a stronger suppressor for the nuclear distribution phenotype of *nudF*6 (Figure 3), which is consistent with the previous report (Willins *et al*., 1997).

The previous genetic study has suggested that the *snfC1232* (*nudAR3086C*) single mutant has a conidiation defect but the *snfC1524* (*nudAL1098F*) mutant has no growth phenotype on its own (Willins *et al*., 1997). Our current results obtained with a genetic
cross and sequencing analyses of selected progeny agreed well with this conclusion but revealed that the nudAR3086C single mutant formed a colony with a slightly reduced size (Figure 2). When the strain carrying nudF6/nudAR3086C was crossed to a wild type strain, progeny of four different sizes appeared when plated at 42°C: the wild type progeny that formed big and healthy colonies, the nudF6 mutant that formed small colonies, the nudF6/nudAR3086C-like colonies that were bigger than the nudF6 mutant but still significantly smaller than the wild type ones, and the nudAR3086C-like colonies that were slightly smaller than the wild type and had a clear conidiation defect (Figure 2). However, when the strain carrying nudF6/nudAL1098F was crossed to a wild type strain, progeny of three different sizes appeared: the nudF6-like and the nudF6/nudAL1098F-like colonies, and the wild-type-like colonies that included both wild type and the nudAL1098F single mutant whose genotypes were confirmed by sequencing analyses. Thus, while the stronger suppressor, nud4AR3086C, causes a clear conidiation defect and a mild colony growth defect, the nudAL1098F mutant has no obvious colony phenotype (Figure 2). It should be pointed out that although the colony phenotype exhibited by the nudAR3086C single mutant was easily detectable, the defect in nuclear distribution pattern was not severe (Figure 3).

3. Neither nudAR3086C nor nudAL1098F suppresses the nud phenotype exhibited by the nudI (dynein intermediate chain) and nudK (Arp1 of the dynactin complex) mutants.

To determine whether these two suppressor mutations specifically compensate for the loss of NUDF or act as general nud suppressors by somehow enhancing the overall
function of dynein, we examined whether these two mutations suppress the phenotype of
nudI (dynein intermediate chain) and nudK (Arp1 of the dynactin complex) mutants.
Dynein intermediate chain and the dynactin complex are implicated in linking dynein to
its membranous cargoes and in increasing dynein motor processivity (Schroer 2004). If
NUDF’s function in dynein regulation differs from that of the dynein intermediate chain
and dynactin, then nudF-specific suppressor mutations should not compensate for the loss
of functions associated with these proteins. We crossed strains carrying GFP-nudA (as a
control), GFP-nudAR3086C and GFP-nudAL1098F respectively to the nudI416 and
nudK317 ts mutants (Xiang et al., 1999; Zhang et al., 2002). The progeny of the crosses
were plated out at the restrictive temperature of 42°C. For the cross between the strain
carrying GFP-nudAL1098F and the nudI416 or the nudK317 mutant, only the nud-like
progeny and the wild type progeny were found, similar to the crosses using the control
strain carrying GFP-nudA. Similar results were obtained with crosses using the GFP-
nudAR3086C strain, except that progeny with even smaller colony size than that formed
by the original nudK317 ts mutant were observed, which was likely due to the additive
effect of the GFP-nudAR3086C and nudK317 mutations. To confirm this result, we
picked eight nud-like progeny from each cross, and analyzed the presence of GFP-NUDA
signals at the permissive temperature. As expected, for the cross between the GFP-
nudAR3086C and nudK317 strains, GFP-NUDA signals were only observed in progeny
that formed smaller colonies than the original nudK317 mutant. For the other crosses,
some nud-like progeny exhibited GFP-NUDA signals while other nud-like progeny did
not. Since the presence of the GFP-NUDA signal is linked to the suppressor mutations,
and the colonies with GFP-NUDA are not bigger than colonies without GFP-NUDA
(Figure 2), these analyses confirmed the notion that neither suppressor mutation suppresses the nud colony phenotype exhibited by the *nudJ416* and *nudK317* mutants. We further examined the nuclear distribution phenotype of progeny containing either suppressor mutation (GFP-NUDA positive) and the *nudJ416* or *nudK317* mutation, and as expected, there was no suppression of the nuclear distribution phenotype (Figure 3).

4. The *nudAR3086C* mutation enhances GFP-dynein’s distribution along cytoplasmic microtubules.

We next examined the effect of these two *nudF* suppressor mutations on dynein’s microtubule-plus-end localization. In the *nudF7/GFP-nudAL1098F* strain, GFP-NUDA formed comet-like structures similar to that in the wild type GFP-*nudA* strain and in the *nudF7/GFP-nudA* strain grown at 32°C (the *nudF7* mutant grows like a wild type at the permissive temperature 32°C). Thus, the *nudAL1098F* mutation did not change dynein’s plus-end accumulation significantly (Supplemental Movies 1, 2 and 3). In contrast, the *nudF7/GFP-nudAR3086C* or the GFP-*nudAR3086C* strain exhibited an obvious alteration in dynein localization. The GFP-NUDA proteins formed small punctates along filament-like structures that most likely represented microtubules (Figure 4, Supplemental Movies 4 and 5). To verify that the signals were indeed along microtubules, we crossed the CFP-*tubA* strain (Li et al., 2005) with the GFP-*nudAR3086C* strain, and found co-localization of GFP-NUDA with CFP-microtubules in hyphal tips (Figure 4). It should be pointed out that GFP-NUDA in wild type cells also formed some small dots along microtubules, but the extent of decoration along the microtubule was much less dramatic compared to that
caused by GFP-*nudAR*3086C. In the Δ*nudF* strain, GFP-NUDA comets seemed longer and more prominent (Figure 4), which is similar to what we have shown previously (Zhang et al., 2003). In the Δ*nudF*/GFP-*nudAR*3086C strain, the comet-like structures could still be seen, but localization along microtubule-like filaments was also found (Figure 4), although in most cells, the signals were less intense compared to that in the GFP-*nudAR*3086C strain.

5. The nudAR*3086C* mutation causes a decrease in the basal level of dynein’s ATPase activity.

To purify *A. nidulans* dynein for biochemical analyses, we have constructed a strain, S-IC, in which the dynein intermediate chain (NUDI) is tagged with the S-tag at its C-terminus (Materials and Methods). By genetic crosses, we have made strains carrying GFP-*nudAR*3086C and GFP-*nudAL*1098F mutations in the S-IC background and these strains are referred to as GFP-*nudAR*3086C/S-IC and GFP-*nudAL*1098F/S-IC respectively. For biochemical analyses, the positive control we used was GFP-*nudA*/S-IC, and the negative control contained just GFP-*nudA* but not the S-IC. After affinity purification using the S-tag-based method (See Materials and Methods), dynein heavy chain could be clearly detected on a silver-stained protein gel (Figure 5A), and a western blot (Figure 5D).

We examined the ATPase activities of dynein isolated from the GFP-*nudAR*3086C/S-IC, GFP-*nudAL*1098F/S-IC, GFP-*nudA*/S-IC and GFP-*nudA* strains. As expected, the S-
peptide-eluted fraction from the GFP-\textit{nudA}/S-IC (positive control) but not that from the GFP-\textit{nudA} strain (negative control) contained significant ATPase activity. In every single experiment (\(n=5\)), dynein with the \textit{nudAR3086C} mutation exhibited a lower basal level of ATPase activity compared to the positive control (Figure 5B). The mean value of dynein’s ATPase activity in the \textit{nudAR3086C} mutant was about half of the wild type value. However, data from the \textit{nudAL1098F} mutant were not consistent with a decreased dynein’s ATPase activity, and the difference between this mutant and wild type is statistically not significant at the \(p\) value of 0.05. Thus, we concluded that the \textit{nudAR3086C} mutation but not the \textit{nudAL1098F} mutation negatively affects dynein’s ATPase cycle.

Under our purification conditions, the S-protein that interacted with the S-tag in the dynein intermediate chain not only was able to pull down the dynein heavy chain, but also was able to pull down the NUDF protein (Figure 5D). Neither \textit{nudAR3086C} nor \textit{nudAL1098F} apparently affected the association between the heavy chain (HC) and intermediate chains (IC) of dynein, and that between NUDF and the dynein complex (Figure 5D). Since IC contains all the light chain binding sites (reviewed by Pfister \textit{et al.}, 2006), we suggested that dynein complex formation is not drastically altered by these mutations.
**Discussion:**

LIS1 and its homologs function in the cytoplasmic dynein pathway in various experimental systems, but mechanistically how they affect the dynein motors in different systems is a question remains to be further explored. In *A. nidulans*, deletion of *nudF* (the *lis1* homolog) produces the same growth and nuclear distribution defect as produced by the dynein heavy chain loss-of-function mutants (Xiang *et al.*, 1995; Willins *et al.*, 1995; 1997). A previous genetic study has suggested that two *nudF* suppressor mutations are located either within or very close to the dynein heavy chain gene, *nudA* (Willins *et al.*, 1997). In this study, we have identified these two point mutations in the dynein heavy chain that partially compensate for the loss of NUDF/LIS1. These two mutations, *nudAL1098F* and *nudAR3086C*, occur in amino acids that are conserved from fungi to human. Thus, further characterization of these mutant forms of dynein in different systems may shed light on how LIS1 and its homologs may regulate the dynein motor.

LIS1 binds to two different sites on the dynein heavy chain, and in addition, it also binds to the intermediate chain of dynein, as well as the dynamitin subunit of the dynactin complex (Sasaki *et al.*, 2000; Tai *et al.*, 2002). Since it binds to both dynein and dynactin, it is possible that LIS1 may mediate dynactin function by facilitating the physical interaction between these two complexes (Tai *et al.*, 2002). In this study, we have shown that the two *nudF* suppressor mutations on the heavy chain do not suppress the phenotypes exhibited by the loss-of-function mutants of dynein intermediate chain and Arp1 of the dynactin complex. Thus, at least some functions of NUDF/LIS1 on dynein regulation are distinct from that of dynein intermediate chain or dynactin. The
LIS1 binding sites on the dynein heavy chain were determined by the yeast two hybrid assay: one in the stem region between amino acids 649-907, and the other is the AAA1 domain (Tai et al., 2002). Whether and how LIS1 affects dynein’s ATPase cycle in vivo is still not clear but an enhancement on dynein’s microtubule-stimulated ATPase activity by purified LIS1 has been detected in vitro (Mesngon et al., 2006). This enhancement on dynein’s motor activity is consistent with LIS1’s positive role in dynein function. However, given our current result that the nudAR3086C mutation decreases dynein’s ATPase activity but partially suppresses the phenotype caused by NUDF loss, it is unlikely that the mechanism of NUDF/LIS1 action on dynein is only related to an enhancement of dynein’s ATPase activity.

The nudAR3086C mutation resides at the end of the AAA4 that is close to the microtubule-binding stalk, but not in the walker A or walker B motif that is implicated in ATP binding or hydrolysis. However, it clearly decreases dynein’s ATPase activity, suggesting that the conformational change caused by this mutation may in turn cause changes in other domains that directly influence dynein’s ATPase cycle. It is not known whether the decrease in the ATPase activity per se is causally related to the suppression. LIS1’s binding site on the heavy chain stem region is close to the sites for heavy chain-intermediate chain and heavy chain-light intermediate chain interactions implicated in cargo-motor binding (Tai et al., 2002), and thus, LIS1 may help to coordinate cargo binding with motor activation (Tai et al., 2002). One may speculate that a decreased ATPase activity may benefit such coordination in the absence of NUDF. Alternatively, a decrease in dynein’s ATPase activity is just a secondary phenotype associated with this
suppressor mutation. Further genetic analyses on more nudF suppressors will be needed to address these issues.

Unlike the nudAR3086C mutation that acts as a stronger suppressor of the nudF deletion phenotype, the nudAL1098F mutation, which acts as a weaker suppressor, does not seem to cause any obvious decrease in the ATPase activity of dynein. Although its suppression on nudF mutants’ nuclear distribution defect is hardly detectable at 42°C, it clearly enhances colony growth of the nudF mutants to some extent, and weakly suppresses the nuclear distribution phenotype of ∆nudF at 32°C. Thus, this mutation may weakly compensate for the absence of NUDF. Given the physical closeness between the nudAL1098F mutation and the deduced LIS1 binding site in the stem, it may be speculated that NUDF/LIS1 binding causes a subtle conformational change of the stem region and the L1098F mutation may partially mimic such a change. Based on deletion analyses and the analysis on trypsin digestion sites, the LIS1 binding site does not seem to be part of the linker domain that connects directly to the motor head domain (Gee et al., 1997; Tai et al., 2002; Hook et al., 2005; Vallee and Hook 2006). Similarly, the A nidulans nudAL1098F mutation, which corresponds to the L1060 residue in human dynein heavy chain, should also reside in the first part of the stem region rather than in the linker domain. A recent EM study has suggested that the stem-to-ring connection is flexible, and the authors speculated that potential regulators such as LIS1 may stiffen the connection to help dynein and its cargo to move forward after a power stroke (Meng et al., 2006). This idea would need further tests. In S. cerevisiae, an artificially constructed dynein heavy chain dimer in which both heavy chains miss the N-terminal regions
including the deduced LIS1-binding sites in the stems as well as the sites for binding other dynein subunits seems to show even more robust physical association with Pac1/LIS1 (Reck-Peterson et al., 2006, supplemental data), suggesting that Pac1/LIS1’s binding to other site(s) may be negatively regulated by part of the stem region. Moreover, this artificial dynein dimer isolated from either the wild type background or the Pac1 deletion background moves on microtubules processively in single-molecule assays, indicating that Pac1/LIS1 is not required for dynein’s processive movement (Reck-Peterson et al., 2006). It remains to be tested, however, whether LIS1 and their homologs have any effect on the motility of native dynein from various organisms. It is worthwhile to point out the recent evidence suggesting that the power stroke of the stem region (or the tail) may drive robust microtubule sliding (Shima et al., 2006).

In contrast to the nudAL1098F mutation that does not seem to cause any apparent change in dynein’s microtubule-plus-end accumulation, the nudAR3086C mutation causes an obvious change in dynein’s localization pattern. In wild type background, GFP-dynein forms comet-like structures representing its localization at the microtubule plus end (Xiang et al., 2000; Han et al., 2001). In some hyphae, small speckles along microtubules can also be found, but it is hard to track them because of the low intensity and the transient nature of these signals. Interestingly, GFP-dynein with the nudAR3086C mutation forms much more speckles along the microtubule (Figure 4). Most speckles seem relatively non-motile, although a time lapse with longer intervals did reveal movements of some speckles (Supplemental Movie 5). Given that the ATPase activity of the nudAR3086C mutant is lower than that in the wild type control, one possibility is that
dynein molecules in this mutant may have difficulty moving towards the minus end of the microtubule. In the ∆nudF background, the nudAR3086C mutation also causes dynein speckles to locate along the microtubule, but in most cells, the decoration seems dimmer compared that in the nudAR3086C single mutant. In many cells, the plus-end comet intensity is not apparently lower than that in the ∆nudF mutant. Thus, although we speculated that the dynein heavy chain fails to interact with microtubule as a minus-end-directed motor in the absence of NUDF, and that the nudAR3086C mutation promotes dynein to leave the plus end by mimicking a conformational change caused by NUDF binding, we have not yet obtained evidence to support this idea. Future studies will be needed to address the mechanism(s) of NUDF/LIS1 action as well as the mechanism(s) of suppression by dynein heavy chain mutations.

In summary, we have identified two nudF suppression mutations in the A. nidulans dynein heavy chain, nudAL1098F and nudAR3086C. Because they are located in different domains and affect dynein differently, we suspect that they may bypass NUDF/LIS1 by different mechanisms. Because these changed residues are conserved from fungi to human, further analyses of dynein with these mutations in several different experimental systems should help to understand how dynein is regulated in the cell. Regarding applying these data to studies in diploid systems, one problem is that we do not yet know whether these mutations are recessive or dominant in terms of nudF suppression since our numerous attempts to make the desired diploids have failed (This study, and Willins et al., 1997). On the other hand, we do know that the nudAR3086C mutation that lowers dynein’s ATPase activity by itself is recessive to the wild type
allele, which makes it harder to study its effect in a diploid system. However, the success in analyzing the ATPase cycle of recombinant dynein in vitro makes it possible to study these recessive mutations in higher systems (Hook et al., 2005; Vallee and Hook 2006), and such studies are likely to generate new insights in the structural-functional relationship of the dynein heavy chain domains.
Acknowledgements:

This paper is dedicated to the memory of Dr. Bo Li. We thank Berl Oakley for sending us \( nKuA \) deletion strains prior to publication, which made this work practically possible. We thank Erika Holzbaur, Steve Osmani and Teresa Dunn for discussions and suggestions, Shihe Li, Liqin Wang and Young Lee for technical help. This work was supported by a National Institutes of the Health grant (GM069527-01), and a Uniformed Services University of the Health Sciences intramural grant (R071GO).
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Table 1 *A. nidulans* strains used in this work*

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<th>Genotype</th>
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*snfC1232 was later identified as *nudA*R3086C; *snfC1524 was later identified as *nudA*L1098F.*
Figure legends:

Figure 1:

Positions of the two nudF suppressor mutations in the dynein heavy chain (nudA) of A. nidulans. (A): A diagram showing the domain organization of the dynein heavy chain. Positions of the nudAL1098F and the nudAR3086C mutations are indicated by red arrows. (B): Sequence alignments of the regions surrounding the nudAL1098F and the nudAR3086C mutations respectively. Accession numbers for human, A. nidulans, N. crassa and S. cerevisiae dynein heavy chains are Q14204, XP_657722, EAA33380 and NP_012980 respectively.

Figure 2:

Growth phenotypes of various nud mutant and nudF suppressor strains at 32°C (A) or 42°C (B, C, and D) for 3 days. (A and B): Both the nudAR3086C and the nudAL1098F mutations suppressed the colony phenotype of the nudF deletion mutant (∆nudF) at 32°C (A) and 42°C (B). Note that the nudAR3086C mutation acted as a strong suppressor at both temperatures. (C): The nudAR3086C mutant had a mild growth defect, but the nudAL1098F did not exhibit any obvious colony phenotype. Note that the nudAR3086C mutation also acted as a strong suppressor for the nudF6 mutant. (D): Neither the nudAR3086C mutation nor the nudAL1098F mutation suppressed the growth phenotypes of the ts nudI416 (in dynein intermediate chain) and nudK317 (in Arp1 of the dynactin complex) mutants. Note that the nudAR3086C mutation even deteriorated the slow-growth phenotype of the nudK317 mutant. In A, B, and D, GFP is linked to nudA in all strains except for the ∆nudA strain.
**Figure 3:**

DAPI staining of various strains. All samples were from 42°C cultures except for the two 32°C samples that are specifically labeled. Bar, approximately 5 µm.

**Figure 4:**

Images of GFP-NUDA in different strain backgrounds showing that the nudAR3086C mutation affects dynein localization. Dynein proteins form comet-like structures representing their microtubule-plus-end localization in wild type cells (wild type). These comet-like structures are more prominent in the ΔnudF mutant (ΔnudF). In the presence of the nudAR3086C mutation, dynein proteins are localized along microtubule-like structures with or without the ΔnudF mutation (ΔnudF/nudAR3086C or nudAR3086C). A merged image is presented to show co-localization of the mutant GFP-NUDA with CFP-microtubule, which is pseudo-colored red (nudAR3086C/MT). Bar, approximately 5 µm.

**Figure 5:**

The ATPase activity of dynein is decreased in the strain carrying the nudAR3086C mutation. (A): A silver-stained gel showing the S-peptide-eluted fractions from strains carrying the S-tagged IC (lane 4 is a negative control without S-IC). Lane 1: nudAL1098C; Lane 2: wild type; Lane 3: nudAR3086C. All these four strains contain GFP linked with nudA. (B): Relative values of dynein’s ATPase activity of the suppressor mutant strains expressed as percentage of the wild type value. Means and standard errors were calculated from multiple experiments (n=5 for wild type and nudAR3086C; n=4 for...
*nudAL1098F*. In every experiment starting from protein purification, two or three samples from the same eluted fraction were subjected to ATPase assays and a mean value was obtained and used for further calculations. (C): Estimated values of dynein’s ATPase activity. These values were from a single experiment with duplicated ATPase assays. The “*” sign indicates that these values are not accurate since the concentration of the dynein heavy chain was estimated from the intensity of a series of BSA samples with different concentrations loaded on the same silver-stained gel. (D). Western blots showing that the levels of dynein HC and NUDF pulled-down by the S-IC are not significantly affected by the suppressor mutations.
Figure 1A

N (0) Stalk 3182-3466 C(4345)
Stem 1-1895 AAA1 1896-2121 AAA3 2554-2803 AAA6 3995-4207
AAA2 2191-2449 AAA4 2897-3166
AAA5 3552-3781

Figure 1B

1048 E Q Y V K V W L Q Y Q C L W D M Q A E N I Y N R L human
1095 G A Y V D K W L Q F Q S L W D L Q S E H V Y D V L N. crassa
1086 S E Y V D K W L Q F Q S L W D L Q S E Q V Y D I L A. nidulans
954 N S Y V K E W Q K M E F L W Q I T E E A F L E V S. cerevisiae

3076 K D R A A T S P A L F N R C V L N W F G D W S T E human
3087 S S K A A T S P A L F N R C V L N W F G D W S D Q N. crassa
3074 S S K A A T S P A L F N R C V L N W M G D W S D Q A. nidulans
2899 S S A M I S S P A L F N R C I I N W M G D W D T K S. cerevisiae
1. a wild type control
2. $\Delta$nudA
3. $\Delta$nudF
4. $\Delta$nudF/nudAR3086C
5. $\Delta$nudF/nudAL1098F
6. a wild type control
7. nudAR3086C
8. nudAL1098F
9. nudF6
10. nudF6/nudAR3086C
11. nudF6/nudAL1098F
12. nudI416
13. nudI416/nudAR3086C
14. nudI416/nudAL1098F
15. nudK317
16. nudK317/nudAR3086C
17. nudK317/nudAL1098F
Figure 4

wild type

ΔnudF/nudAR3086C

nudAR3086C

nudA
R3086C/MT
Figure 5

A

B

C

D

ATPase activity (% of the wild type value)

ATPase activity (µmol/minute/mg)

wild type  nudAR3086C  nudAL1098F

wild type  nudAR3086C  nudAL1098F

HC
S-IC
NUDF

1  2  3  4