The Nuclear Migration Protein NUDF/LIS1 Forms a Complex with NUDC and BNFA at Spindle Pole Bodies

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Nuclear migration depends on microtubules, the dynein motor complex, and regulatory components like LIS1 and NUDC. We sought to identify new binding partners of the fungal LIS1 homolog NUDF to clarify its function in dynein regulation. We therefore analyzed the association between NUDF and NUDC in Aspergillus nidulans. NUDF and NUDC directly interacted in yeast two-hybrid experiments via NUDF’s WD40 domain. NUDC-green fluorescent protein (NUDC-GFP) was localized to immobile dots in the cytoplasm and at the hyphal cortex, some of which were spindle pole bodies (SPBs). We showed by bimolecular fluorescence complementation microscopy that NUDF directly interacted with NUDC at SPBs at different stages of the cell cycle. Applying tandem affinity purification, we isolated the NUDF-associated protein BNFA (for binding to NUDF). BNFA was dispensable for growth and for nuclear migration. GFP-BNFA fusions localized to SPBs at different stages of the cell cycle. This localization depended on NUDF, since the loss of NUDF resulted in the cytoplasmic accumulation of BNFA. BNFA did not bind to NUDC in a yeast two-hybrid assay. These results show that the conserved NUDF and NUDC proteins play a concerted role at SPBs at different stages of the cell cycle and that NUDF recruits additional proteins specifically to the dynein complex at SPBs.

Nuclear movement is a precisely regulated developmental process that is of special importance in highly elongated cells, like neurons or the linear hyphae of filamentous fungi. These cell types grow by apical extension and the subsequent long-range translocation of nuclei into the leading process. In neurons, this nuclear movement can be followed by the retraction of the trailing process so that active cell migration, so-called nucleokinesis, is achieved (37).

Important aspects of nuclear migration were elucidated by genetic analyses performed with the amenable fungal model organism Aspergillus nidulans. In a screen for nuclear migration mutant strains, the genes nudA, nudC, nudF, and nudG were discovered (60). The human homolog to nudF is Lis1, the haploinsufficiency of which causes autosomal-dominant lissencephaly type 1 (10). Mutations of Lis1 lead to retarded neuronal migration to the cerebral cortex, creating disorganized cortical layers. This defective brain development causes mental retardation, epilepsy, and early death. The characterization of LIS1-interacting proteins showed that LIS1 also plays a role in other diseases, like schizophrenia and neuronal degeneration (45). While during the nucleokinesis of neurons a single nucleus is transported through the cytoplasm, fungal growth is characterized by the distribution of multiple nuclei along filamentous hyphae. Mutations of nudF prevent the active transport of nuclei out of the spore into the growing hyphae, leading to the accumulation of nuclei near the spore remnant and only incidental nuclear motion, which leads to slow growth and excessive hyphal branching (61). In addition, nuclear migration is important for the development of asexual spores in A. nidulans, during which, after several rounds of nuclear division and the budding of specialized cells (sterigmata), uninucleate and green spores are finally released. Without NUDF, mainly anucleate sterigmata are formed, and conidia are generated only if a nucleus occasionally enters a bud (61). Thus, nuclear migration mutants of A. nidulans are easily recognized by their knobby, brownish colony appearance due to slow growth and the reduced production of colored spores. NUDF/LIS1 is a 50-kDa dimeric protein with an N-terminal α-helical LisH dimerization motif, a coiled-coil helix, and a C-terminal WD40 domain, which is a β-propeller (29, 53).

The nudA and nudG nuclear migration mutations of A. nidulans that show the same phenotype as the nudF mutation affect genes encoding the heavy and light chains, respectively, of the microtubule (MT)-dependent motor dynein (60). NUDF is not a subunit of cytoplasmic dynein, but it regulates dynein motor function. Dynein heavy-chain mutations were isolated as extragenic suppressors of a temperature-sensitive nudF mutation (57) and subsequently were shown to affect an ATPase domain of dynein and its stem region (66). NUDF directly bound the first ATPase domain of the dynein heavy chain in a yeast...
two-hybrid analysis (48). Accumulated in vivo data suggest that NUDF is necessary for activating dynein’s retrograde transport activity, and without NUDF, dynein accumulates at MT plus ends (63). Dynein heavy chain and NUDF were colocalized at MT plus ends and at spindle poles and influence the stability of MTs by their availability at plus ends (35). Mutations in the MT plus ends and at spindle poles and influence the stability of ends (63). Dynein heavy chain and NUDF were colocalized at MT plus ends of dividing nuclei attached to the MT minus ends can be brought to and fixed at specific cellular positions (56).

In addition, various other nud mutants have been described that regulate NUDF function. NUDF is a multicopy suppressor of the nudF7 mutation and has been shown to interact with NUDF’s coiled-coil-helix via its own N-terminal coiled-coil domain (13). Its C-terminal domain directs NUDF to MT plus ends, but NUDF also forms immobile specks along hyphae, suggesting additional cortical functions of NUDF (14). Whereas it has been shown that NUDF is recruited to MT plus ends by CLIPA and NUCED (14), it is unknown whether these proteins also are responsible for targeting NUDF to spindle poles. CLIPA belongs to the group of plus-end tracking proteins. The mammalian homolog CLIP-170 also regulates MT dynamics and mediates MT capture at cortical sites (20, 31). Furthermore, NUDF is stabilized by NUDC, and the level of NUDF is reduced in the nudC3 mutant, indicating that the other nud mutants, a nudC deletion in A. nidulans results in the loss of polar growth, abnormal cell walls, and the lysis of cells, which hints at additional essential functions (8).

Mammalian NUDC contains an extra N-terminal domain in addition to a conserved p23 domain. Murine NUDC interacts biochemically with LIS1 and copurifies with the dynein heavy and intermediate chains. Similarly, the colocalization of murine NUDC, LIS1, and dynein at MT-organizing centers near the nucleus indicate that NUDC exerts its function at least partly via the regulation of the dynein/LIS1 complex (38). In addition, murine NUDC was found at discrete foci at the cortical cytoskeleton. In different rat cell types, NUDC is localized at the region of the Golgi apparatus (39). Human NUDC (hNUDC) is essential for bipolar spindle formation, indicating a function in MT organization at spindle poles (64). hNUDC also is localized to the kinetochores and regulates MT attachment to chromosomes (41). During late mitosis, it is found at midzone MTs and the midbody in HeLa cells, which emphasizes its role for cytokinesis (1). Thus, NUDC plays a broad role during mitosis, which is in agreement with its high level of expression in proliferating cells. A mitotic function also can be attributed to LIS1. Together with dynein, it is localized at the cell cortex, the centrosome, and mitotic kinetochores, which also regulate spindle orientation, chromosome attachment, and the cortical tethering of astral MTs (16, 52). Unlike the case for Aspergillus, LIS1 overexpression in mammalian cells blocks mitotic progression (16), while the complete inhibition of mitotic entry by the reduction of LIS1 expression was observed for noneural mammalian cells (55).

In this study, we intended to identify new binding partners of the fungal LIS1 homolog NUDF. We analyzed where NUDC is localized in A. nidulans and if there are physical interactions between fungal NUDC and NUDF. Furthermore, we asked whether unknown NUDF binding proteins can be identified in this filamentous fungus in order to gain further insights into the molecular function and regulation of this nuclear migration protein.

MATERIALS AND METHODS

Strains, media, and growth conditions. The Aspergillus nidulans strains used in this study are listed in Table 1. Escherichia coli strains DH5α (59) and SURE (Stratagene, Amsterdam, The Netherlands) were employed for the preparation of plasmid DNA and were grown in Luria-Bertani (LB) medium (1% tryptophan, 0.5% yeast extract, 1% NaCl) in the presence of 100 μg/ml ampicillin. The bacterial strain KS272 carrying the pKOBEG plasmid was grown in LB medium in the presence of 25 μg/ml chloramphenicol (7). Minimal medium (MM; 1% glucose, 2 mM MgSO4, 70 mM NaNO3, 7 mM KCl, 11.2 mM KH2PO4) 0.1% case element solution [28], pH 5.5, 2% agar) and YAG medium (2% glucose, 0.5% yeast extract, 2% agar) either with or without 0.6 mM KCl were used for the growth of Aspergillus strains and were supplemented with the appropriate amounts of pyridoxine-HCl (0.1%), uridine (5 mM), and uracil (5 mM) for agar plates. For two-hybrid experiments, Saccharomyces cerevisiae strain EGY48 (MATa trp1 his3 ura3-52 leu2-3,112 trp1 his3 leu2-3,112 ade2-1) harboring pPR1840 (URA3 leu2-3,112 trp1-248 ura3-52 trp1 his3 leu2-3,112 ade2-1) was grown on selective synthetic complete (SC) medium (0.15% yeast nitrogen base, 0.55% (NH4)2SO4, 0.1% (vol/vol) 200 mM mgso-inositol, 0.2% amino acid mix-4, 2% glucose, 2% agar) lacking tryptophan, uracil, histidine, and, in the case of growth assays, leucine (SC-3 and SC-4). When needed, supplements were added according to Guthrie and Fink (24).

Transformation procedures. E. coli cells were transformed as described previously (27) or by applying electroporation (50) with a Bio-Rad Gene Pulser at 2.5 kV in 0.2-cm cuvettes (Bio-Rad Laboratories GmbH, Munich, Germany). A. nidulans was transformed by the polyethylene glycol-mediated fusion of protoplasts as described previously (43). S. cerevisiae was transformed by a modified method based on that of Elbe (15).

Plasmid and strain constructions for TAP. A nudF deletion strain was generated for the reintegration of a tandem affinity purification (TAP)-tagged nudF version for expression from the authentic promoter. For that purpose, a deletion cassette containing 250-bp 5′-untranslated region (5′ UTR) and 3′ UTR fragments of nudF was generated as described by Krapmann et al. (32) using the zeo-ArgpG-zeo deletion cassette for targeted gene replacement. A 10-kb genomic BglII fragment, containing nudF, was amplified from an A. nidulans strain AGB257 containing an artificial chromosome library by using a 640-bp probe, which was amplified using the HexaLabel DNA labeling kit (Fermentas GmbH, St. Leon-Rot, Germany) and hybridized to the 5′ UTR of nudF. The genomic fragment was subcloned into BamHI-digested pBluescript (pME2822). This plasmid and the nudF deletion cassette were transformed into E. coli K12S272, and the plasmid resulting from homologous recombination (pME3231) was rescued from K12S272. It contained the nudF::zeo::pyrG::zeo deletion cassette with a 6-kb 5′ UTR and 3-kb 3′ UTR and was linearized by NotI digestion prior to transformation into A. nidulans strain AGB152, yielding strain AGB257 (nudF::zeo-ArgpG::zeo pyrG89 pyrA4). Transformants were selected on YAG medium containing pyridoxine-HCl and 0.6 M KCl and were tested for their lack of nuclear migration by the staining of nuclei, which was performed as described previously (60), and for small-colony growth by growth tests on MM pyridoxine plates and was compared to that of the parental strain. The pyrG marker of AGB257 was rescued by being plated on 5-fluoroacetic acid (5-FOA) medium according to Krapmann et al. (32), resulting in strain AGB294.

A TAP-tagged version of nudF was reintegrated into the nudF locus of AGB257. An Muid site was created 5′ of the start codon of nudF in pME2822, which was employed for the integration of the N-terminal TAP tag with optimized codons and without an internal Muid site (ntup+a; pME3232). This plasmid was digested with NotI and transformed into AGB257 with selection on MM containing sorbitol, pyridoxine-HCl, uracil, uridine, and 1 mg/ml 5-FOA, resulting in strain AGB249 (nTAP+::nudF pyrG89 pyrA4). Again, transformants were controlled by the microscopic analysis of nuclear migration and by growth tests, which proved the functionality of the tagged NUDF.

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TABLE 1. Aspergillus nidulans strains used in this study

| Strain   | Genotype                | Source or reference         |
|----------|-------------------------|----------------------------|
| AGB152   | pyrA4 pyrG89            | 5                          |
| A1149    | pyrA4 pyrG89 avkA::argB  | This study                 |
| A779     | nudC3 wA2 nicA2 pabaAI pyrG89 | FGSC (Kansas City, MO)    |
| XX20     | nudF6 pyrG89            | 61                         |
| AGB241   | pabaAI nicA2 wA2 pyrG89/ pyr-4 nudC3 7核酸检测: nudC::gfp | This study                 |
| AGB249   | nATP::nudF pyrG89 pyrA4  | This study                 |
| AGB257   | pyrA4 pyrG89 avkA::argK | This study                 |
| AGB294   | pyrA4 pyrG89 avkA::argB  | This study                 |
| AGB295   | pyrA4 pyrG89 avkA::argB  | This study                 |
| AGB296   | pyrA4 pyrG89 avkA::argB  | This study                 |
| AGB297   | pyrA4 pyrG89/4 nudC::zeo gpda::natR 7核酸检测: nudC::gfp2-5::natA::gfp | This study                 |
| AGB298   | pyrG89/pyr-4 nudF6 pyrG89/7核酸检测: nudC::gfp2-5::natA::gfp | This study                 |
| AGB299   | pyrA4 pyrG89/4 nudC::zeo gpda::natR 7核酸检测: nudC::gfp2-5::natA::gfp | This study                 |
| AGB300   | pyrA4 pyrG89/4 nudC::zeo gpda::natR 7核酸检测: nudC::gfp2-5::natA::gfp | This study                 |
| AGB301   | pyrA4 pyrG89/4 nudC::zeo gpda::natR 7核酸检测: nudC::gfp2-5::natA::gfp | This study                 |
| AGB303   | nudG89/4 nudC::zeo gpda::natR 7核酸检测: nudC::gfp2-5::natA::gfp | This study                 |
| AGB334   | nudG89/4 nudC::zeo gpda::natR 7核酸检测: nudC::gfp2-5::natA::gfp | This study                 |
| AGB335   | nudG89/4 nudC::zeo gpda::natR 7核酸检测: nudC::gfp2-5::natA::gfp | This study                 |
| AGB336   | nudG89/4 nudC::zeo gpda::natR 7核酸检测: nudC::gfp2-5::natA::gfp | This study                 |
| AGB337   | nudG89/4 nudC::zeo gpda::natR 7核酸检测: nudC::gfp2-5::natA::gfp | This study                 |
| AGB338   | nudG89/4 nudC::zeo gpda::natR 7核酸检测: nudC::gfp2-5::natA::gfp | This study                 |

Plasmid and strain constructions for bnf4 deletion. The bnf4 knockout strain was generated by applying the zeo-Afpyr-G::zeo deletion cassette. A 986-bp 5′ UTR and a 976-bp 3′ UTR fragment were PCR amplified and cloned into the EcoRV and HpaI restriction sites of pME2049, respectively (OLKH241, 5′-ATC GTG ATT CTC TAT TGG ATC CGC-3′; OLKH242, 5′-GGA GCT GGC TGG AGA TCG-3′; OLKH243, 5′-GAC TCG CAA TAA GAG TAC AC-3′; and OLKH244, 5′-AAC CGT TTT CAT TGG ACC CGC AGT-3′), resulting in plasmid pME3233. The linearized plasmid was transformed into the Dnak ad strain A1149, and transformants were selected on YAG medium containing pyridoxine-HCl and 0.6 M KCl. The pyrG marker was rescued by being plated on 5-FOA medium, resulting in strain AGB295. Since the backcrossing of these Dnak ad strains was not successful, the Dnak ad::zeo deletion also was generated in the AGB152 strain background (AGB296), and the last one was used for localization experiments.

Plasmid and strain constructions for localization experiments. NUDC-green fluorescent protein (NUDC-GFP) was localized in an A. nidulans nudC ad strain after the transformation of pME823 harboring a nudC::gfp fusion into A779 (AGB241). pME823 was created by cloning a 7kb fragment of nudC into pmBC32 (18) (OLKH115, 5′-AAG GTA CCA TCA TGG ACC AAC CGT C-3′; and OLKH116, 5′-AAG GTA CCA TCA TGG ACC AAC CGT C-3′).

For the colorization of NUDF and NUDC, the encoding genes were fused into the Dnak ad strain A1149, and transformants were selected on YAG medium containing pyridoxine-HCl and 0.6 M KCl. The pyrG marker was rescued by being plated on 5-FOA medium, resulting in strain AGB295. Since the backcrossing of these Dnak ad strains was not successful, the Dnak ad::zeo deletion also was generated in the AGB152 strain background (AGB296), and the last one was used for localization experiments.

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KpnI fragment with nudF coded into a pMCI17apx derivative (pME3322). In some transformatants, this plasmid did not integrate ectopically but did so solely at the nudF locus, resulting in a gfp-z labeled nudF copy driven by the autonomous promoter and an unlabeled copy driven by the alc4 promoter from the plasmid. Therefore, this strain (AGB338) was grown in glucose medium for microscopic analysis so that no unlabeled NUDF was present in the cells.

For the localization of NUDF in the A. nidulans strain, nudF was cloned into pMCI17apx via AscI/PacI (OLKH272, 5'-AGG CGG CCC GAC CAA ATT GAC AGC TCC-3' and OLKH273, 5'-CTA TAA TTA ATT AGC TGA ACA CCC GTA CAG-3'), and a c-terminally fused 244 amino-acid fragment (fused to a NotI/SmaI fragment with tubA (OLKH274, 5'-CTA AGA ATG CGG CCC GAG AGT AGT TAG TTG GAT TAA CGG-3'), or Phusion high-fidelity DNA polymerase (Finnzymes/New England Biolabs GmbH, Frankfurt am Main, Germany) or PfuTurbo DNA polymerase (Stratagene, München, Germany) were used in vector pME3324 and pME3325, encoding aa 1 to 28, was amplified with primers OLKH147 (5'-GCC GGG CCC GGG GTA TTC TAA TTA ATT AGC TGA-3'), and OLKH148 (5'-CGG GAA TCA TTC CAA ACT CGT ATT CGG-3') and was cloned into pME2938. The negative control, vector pME2938 was used in combination with empty vector pME3324.

For the domain interaction analysis, the LiH motif-coiled-coil region of NudF (amino acids 1 to 28) was amplified with primers OLKM3 (5'-CCC CTC GAG TATG AGC ATG AGC CAA ATG TAC GCT C-3') and OLKM4 (5'-CCC CTC GAG CAC CAA TATG AGC GCC CAG-3') and was cloned into pME3252. The resulting plasmid, pME3255, encoding the first 585 bp of nudF, was cloned into a pMCB17apx derivative (pME3323). As a positive control, the first 585 bp by encoding the first 850 bp of nudF, was cloned into pME2938. Therefore, this strain was used in combination with empty vector pME3324. Western hybridization experiments were performed for expression analysis using anti-hemagglutinin (Sigma-Aldrich Chemie GmbH, Munich, Germany) and anti-LexA antibodies (Santa Cruz Biotechnology, Inc., Heidelberg, Germany), respectively.

For interaction tests, overnight SC cultures were diluted to an optical density of 546 nm of 0.2 with SC medium, and 10 μl of the dilution was spotted onto SC-3 plates containing 2% galactose–1% raffinose or 2% glucose (negative control) for growth tests. Dilutions at an optical density of 546 nm of 0.1 were spotted onto SC-3 plates containing 2% galactose–1% raffinose and 1.7 mM leucine for subsequent β-galactosidase filter assays. Plates were incubated at 30°C for 2 days. For the filter assay, a Whatman filter was placed onto the yeast plate, shock frozen in liquid nitrogen, and dried. The filter then was put onto autoradiograph paper with 3 ml of Z buffer (60 mM Na2HPO4, 20 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4, 0.1% X-Gal) and incubated at 30°C for 4 h.

Microscopy. Five hundred microliters of the appropriate medium was put onto coverslips, which were placed in petri dishes and inoculated with 4 × 10⁴ spores. For growth at 42°C, coverslips were placed into 6-well plates with 5 ml of medium.
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inoculated with $2 \times 10^5$ spores or into petri dishes with 20 ml of medium inoculated with $1 \times 10^6$ spores. After incubation at the respective temperatures, slides were mounted on glass slides with nail polish. For the staining of nuclei, 1 μl of 4',6'-diamidino-2-phenylindole (DAPI) was spotted onto the glass slide before placing the coverslip onto it. Cells were examined with a Zeiss 100 Axiosvert microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany), and photungstic acid solution, pH 7.0. Specimens were analyzed with a Philips EM 301 instrument (Philips, Hamburg, Germany) at calibrated magnifications and using IMAGO electron-sensitive films (Atomic Force F&E GmbH, Mannheim, Germany). Immuno-gold labeling was performed as described earlier (26).

For electron microscopy, embedding in Lowicryl K4M resin was performed as described previously (26, 46). Resin sections of about 80 nm in thickness were cut with glass knives. The sections were stained for 3 min with 3% [wt/vol] phosphotungstic acid solution. Cells were examined with a Philips EM 301 instrument (Philips, Hamburg, Germany) at calibrated magnifications and using IMAGO electron-sensitive films (Atomic Force F&E GmbH, Mannheim, Germany). Immunogold labeling was performed as described earlier (26).

In silico analyses. For manual annotations, we used the genome databases of CADRE (www.cadre-genomes.org.uk), TIGR (www.tigr.org/tdb/c2k1/afu1/), the Broad Institute (www.broad.mit.edu/annotation/fungi/fgi/), JGI (genome.jgi-psf.org), and the Center for Integrated Fungal Research (www.aspergillusflavus.org). Domain analyses were performed using MyHits (myhits.isb-sib.ch/cgi-bin/motif_scan) and the COILS server (www.ch.embnet.org/software/COILS_form.html). Protein alignments were carried out with NPSA (npsa-phil.ibe.pf.fr/cgi-bin/npsa_automat.pl?page=npsa_clustalw.html).

RESULTS

NUDF is localized to immobile dots at the cell cortex. Mammalian NUDC was described to interact and colocalize with LIS1, while fungal NUDC was only shown to stabilize NUDF posttranslationally (61). Therefore, we intended to characterize the fungal NUDC in more detail. As a first step toward deciphering its molecular function, we localized NUDC in A. nidulans. We expressed a "alaA::nudC::sgfp" fusion in the nudC3 strain (AGB241) and confirmed the functionality of the fusion protein by the complementation of the temperature-sensitive nudC3 phenotype by growing AGB241 at 42°C (data not shown). Fluorescence microscopy was performed after the germination of spores in glycerol-containing medium at 42°C. NUDC-GFP was observed as immobile dots along hyphae that were obviously near the cytoplasmic membrane (Fig. 1A). We also conducted immunoelectron microscopy for a more detailed view of the NUDC-GFP position. In fact, NUDC-GFP was detected at the hyphal cortex, with isolated spots in the cytoplasm (Fig. 1B). In the enlarged tip section, NUDC also decorated the cortex around the hyphal tip. Thus, the majority of NUDC obviously localized differently from NUDF, which was detected at spindle poles and MT plus ends, but rather resembled the immobile specks observed for NUDGE (12).

NUDF associates with NUDC at spindle pole bodies (SPBs) and at the cortex. Although at first sight NUDF and NUDC localized at different sites in A. nidulans, the colocalization of their homologs in mammalian cells prompted us to pursue the investigation of the putative interaction between the Aspergillus proteins. We analyzed their association in a yeast two-hybrid assay based on LexA and B42 fusions, respectively. The B42-NUDF fusion protein was not correctly expressed, so it could not be detected by Western hybridization (data not shown). The LexA-NUDC fusion was detected by Western hybridization and associated with the B42-NUDE fusion, as indicated by the expression of the LEU2 and lacZ reporter genes (Fig. 2B). Furthermore, we wanted to specify by which domains of NUDF and NUDC the attachment is accomplished. Based on analyses with computer programs and its similarity to p23 (21), NUDC was predicted to consist of an N-terminal coiled-coil helix that replaces a large coiled-coil domain of mammalian NUDC, a p23-like central domain (a β-sandwich), and an unknown C-terminal domain of 83 aa.

FIG. 1. NUDC is localized to immobile dots at the A. nidulans hyphal cortex. Fluorescence microscopy (A) and immunoelectron microscopy (B) of strain AGB241 showed that NUDC-GFP dots are positioned along the hyphal cortex and the tip (examples are indicated by arrowheads). The strain was grown in MM containing 2% ethanol at 42°C for 15 h. Scale bars, 5 

| A | DIC | NUDC-GFP | DIC | NUDC-GFP |
|---|-----|----------|-----|----------|
| B | cp  | cw       | cp  | cw       |
| C | DIC | MIPA-mRFP | NUDC-GFP | merge |

Scale bars, 5 

DIC, differential interference contrast.
NUDF presumably contains an N-terminal LisH dimerization motif followed by a coiled-coil helix and a large WD40 domain (Fig. 2A) (29, 53). The parts of the ORFs encoding these domains were fused to the respective two-hybrid domains and expressed in yeast for comparisons to the full-length ORFs. The growth assay as well as the β-galactosidase filter assay revealed an interaction between the full-length NUDC and the WD40 domain of NUDF (Fig. 2B). In the growth assay, a presumably false-positive interaction was detected between the coiled-coil domains of NUDF and NUDC through the expression of the more sensitive LEU2 reporter construct. No interactions were observed involving the separate p23 and C-terminal domain of NUDC, respectively, although all fusion proteins were detected by Western hybridization (data not shown). Whether the single domains do not adopt the correct fold when fused to the two-hybrid domains or whether the domains must form a specific quaternary structure that then can bind to NUDF's WD40 domain cannot be resolved at the moment. Thus, using two-hybrid analysis, we could not identify the interacting domain of NUDC.

However, these results made us analyze the NUDF-NUDC interaction in vivo. For that purpose, we constructed fusions with the N-terminal and C-terminal halves of eYFP to observe bimolecular fluorescence complementation (BiFC) in case both fusion proteins were in close contact with each other (25).
The fusions were expressed from the bidirectional *niaA/niaD* promoter by growth on nitrate-containing medium and partially complemented the temperature-sensitive phenotypes when expressed in the *nudC* and *nudF* strains (*AGB302* and *AGB303*, respectively). These strains grew faster and produced more conidia than the temperature-sensitive parental strains at 42°C but did not grow as fast at 30°C, which indicated the partial functionality of the NUDF-yEYFP and NUDC-cEYFP fusions (data not shown). Although the YFP emission was low, numerous dots were detected along hyphae and at nuclei that were labeled by the constitutive expression of an *mrfp*::*h2A* fusion (Fig. 2C). Fluorescence also was detected at both poles of mitotic nuclei (Fig. 2C), showing that NUDF-NUDC interaction also takes place at SPBs during mitosis.

These sites were clearly identified as SPBs by colocalization with γ-tubulin (MIPA), which was fused to monomeric RFP (mRFP) in strain AGB335 (Fig. 2D). In this strain, a few prominent spots were observed among several fainter fluorescent spots in the cytoplasm, and the prominent spots clearly could be allocated to the mRFP-labeled SPBs. The SPBs often jerked in the cells but also were found immobilized near the cortex, in which case accurate superimposition with BiFC spots could be best achieved.

To investigate whether some of the dots observed for NUDC-GFP alone also colocalized with SPBs, we introduced a *nudC::gfp* fusion into a strain harboring the *mipA::mrfp* fusion (*AGB338*) (Fig. 1C). In addition, *nudC* was not overexpressed but was expressed from the authentic promoter, and it was fused to the more stable *gfp2-5* version. In fact, NUDC-GFP spots could be colocalized with MIPA-mRFP signals even in mitotic nuclei (Fig. 1C, lower row).

**BNFA is a novel protein that physically interacts with NUDF in A. nidulans.** Since several aspects of NUDF localization and the regulation of its activity are still unclear, we searched for additional, unknown interaction partners of NUDF and performed TAP using an nTAP*-tagged NUDF. The use of this tag allowed two subsequent affinity purifications, the first on IgG Sepharose due to the presence of two protein A domains and a second on calmodulin resin in the presence of Ca²⁺ by virtue of a calmodulin binding epitope (CBP) directly N terminal to NUDF. Both tags were separated by a TEV cleavage site so that CBP-NUDF could be cleaved off the first matrix by incubation with TEV protease. Elution from the calmodulin beads was achieved through titrating out calcium by the addition of EGTA.

The fusion protein was expressed from the authentic *nudF* promoter by the reintegration of the *nTAP*::*nudF* fusion (*AGB249*) into the *nudF* locus of a Δ*nudF* strain (*AGB257*). The functionality of the fusion protein was confirmed by its complementation of the nuclear migration and growth defect.
of the nudF deletion (Fig. 3A). DAPI staining of nuclei showed that the even distribution of nuclei along hyphae was restored in the transformed strain AGB249, although a few nuclei accumulated in the remainder of the conidia. Nevertheless, the nearly full functionality of the fusion protein enabled this strain to grow like the parental strain AGB152 (wild-type nudF).

*A. nidulans* crude extracts were prepared from 18-h-old vegetative mycelia. The TAP was monitored by taking samples at critical steps of the procedure and detecting the NUDF fusion protein with an anti-CBP antibody in a Western hybridization (Fig. 3B). Proteins in the final eluate that bound to the IgG and calmodulin matrices, respectively, were analyzed by SDS-PAGE and silver staining (Fig. 3C). Proteins binding unspecifically to the beads could be identified by TAP using the crude extract of the parental strain AGB152, which did not express a tap* fusion but instead expressed native nudF (Fig. 3C). Several high-molecular-weight proteins were present in both crude extracts, indicating that they were not copurified with NUDF but bound to the beads directly. The lower three bands in the lane with proteins from strain AGB249 were cut out and analyzed by tandem mass spectrometry after trypsin digestion. The upper band was identified as NUDF (AN1697.1; two tryptic peptides of the protein with cross correlation [Xcorr] values of 3.5 and 3.9) and the band below as the elongation factor 1A α-subunit (AN4218.1; three peptides with Xcorr values of 2.3, 3.0, and 3.4). The elongation factor also was identified in other control purifications, and since it was shown to bind to calmodulin (11), its copurification in this experiment probably was not due to a specific binding to NUDF. The lower band contained the gene product of ORF AN3213.1 (only one peptide, with an Xcorr value of 2.8), which we called BNFA.

BLAST analyses showed that BNFA did not bear similarities to any known protein. Therefore, manual annotation regarding the AN3213 locus and its neighboring loci (AN3211, AN3212, and AN3214) was performed using the genomes of *Aspergillus fumigatus*, *Aspergillus oryzae*, *Aspergillus terreus*, *Aspergillus niger*, and *Aspergillus flavus*. A BLAST search of the A. niger genome identified locus 669910-670782 on scaffold 8 (protein identity 41676). The encoded protein bears 27% identity and 53% similarity to BNFA, indicating a divergent protein even in closely related species. The synthesis of this genomic region was confirmed by the identification of genes homologous to AN3212.3 and AN3211.3, respectively, on scaffold 8 (loci 672614 to 673576 and 674136 to 674961). In the other *Aspergillus* species, no bnfA homolog could be detected. In *A. terreus*, the genes homologous to AN3212.3 and AN3211.3 were located next to each other (ATEG_07921.1 and ATEG_07922.1). In *A. fumigatus*, four other genes were inserted between the genes homologous to AN3212.3 and AN3214.3 (Afu4g01060-30), and in *A. oryzae* and *A. flavus* the neighboring loci were scattered along the genome. Thus, not only is BNFA a divergent protein but also the whole genomic region is highly variable. Therefore, BNFA presumably exists but still is untraceable in other organisms.

Domain analyses of BNFA indicated the presence of proline-rich regions spanning aa 11 to 85 and 238 to 297 and a C-terminal coiled-coil region between aa 302 and 350 (Fig. 4). The similar hypothetical protein of *A. niger* also was predicted to form a C-terminal coiled-coil region (aa 250 to 287), but it showed gaps in the proline-rich regions of BNFA. The strongest similarities between BNFA and the hypothetical *A. niger* protein were found in the central part located between these regions.

The novel physical interaction of NUDF with BNFA in *A. nidulans* was confirmed genetically by yeast two-hybrid analysis. According to the genome sequence, in which there is no intron, a 1,065-bp coding sequence could be PCR amplified for bnfA from the cDNA library pCNS4 and was cloned into yeast two-hybrid vectors containing ORFs for the B42 activation domain and the LexA DNA-binding protein. The known interaction between NUDF and the coiled coil of NUDF was used as a positive control. The b42-bnfA fusion impaired the growth of *S. cerevisiae* strain EGY48 (data not shown), so the test for leucine prototrophy could not be performed. However, lacZ reporter gene expression was induced in EGY48 harboring the b42-bnfA fusion plus a lexA-nudF fusion, confirming the interaction between NUDF and BNFA (Fig. 3D). The expression of a lexA-bnfA fusion did not result in retarded growth (data not shown), and no interaction with B42-NUDF was observed in the β-galactosidase assay (Fig. 3D) due to the fact that this fusion protein was not properly expressed. No fusion protein was detected by Western hybridization (data not shown).

**BNFA is located at *A. nidulans* SPBs in a NUDF-dependent manner.** We deleted bnfA in order to investigate the cellular function of this novel protein. In the ∆nkuA strain A1149 and in AGB152, the bnfA ORF was replaced by the zeo::pyrG::zeo blaster cassette with subsequent counterselection against pyrG. However, no nuclear migration phenotype was observed. DAPI staining of nuclei in germinating hyphae demonstrated an even distribution, and in growth assays a wild-type-like growth rate and asexual development were observed in both strain backgrounds (data not shown).

A gfp2-5::bnfA fusion was expressed in the ∆bnfA strain AGB296 to gain insight into BNFA’s cellular role through localization experiments. Again, a prominent nuclear dot pattern was observed in germinating hyphae characteristic of SPB proteins (Fig. 5A). In addition, a faint fluorescence was detected throughout the nuclei, which could indicate a role for BNFA in the nucleus, but it also might be caused by the rather
high expression from the alcA promoter during growth on glycerol-containing medium. As a control, nuclei were counterstained by the expression of an mrfp::h2A fusion (Fig. 5A). In several cases, we observed two dots of GFP-BNFA in close proximity to each other, indicating that the respective nuclei were undergoing mitosis, during which the SPB was duplicated for spindle formation (Fig. 5A). For clarification, a bnfA::gfp2-5 fusion was expressed from the bnfA promoter in a strain with labeled SPBs (AGB337). Microscopy revealed the same fluorescent pattern within the cells, which was weaker due to the lower expression level, but it also corresponded to the γ-tubulin pattern. Again, an additional faint fluorescence was observed throughout nuclei, so that an overexpression effect could be ruled out.

Thus, BNFA could be detected at SPBs at different stages of the cell cycle. This result corroborated the NUDF-BNFA interaction, since YFP-NUDF also was localized to SPBs, albeit only during mitosis (35). These findings lead to the question of whether NUDC also interacted with BNFA. However, no association between the LexA-NUDC and B42-BNFA fusion proteins could be detected in a yeast two-hybrid assay (data not shown).

BNFA might be an integral part of the SPB recruiting NUDF to the nucleus, or BNFA might be placed at the SPB by means of NUDF. Therefore, GFP-NUDF was localized in the ΔbnfA strain and compared to the wild-type bnfA background (AGB300 and AGB301) to discriminate between these possibilities. GFP-NUDF was localized at MT plus ends during interphase and at the poles of mitotic nuclei, indicating that BNFA is not necessary for recruiting NUDF to SPBs (data not shown). The GFP-BNFA localization then was analyzed in the absence of NUDF. The gfp2-5::bnfA and mrfp::h2A fusions were expressed in a temperature-sensitive nudF6 strain (AGB298), and fluorescence microscopy was performed after growth in glycerol-containing medium at room temperature and 42°C, respectively. At low temperature, GFP-BNFA localized to SPBs as seen before (data not shown). At 42°C, the typical nuclear migration defect could be observed with nuclei clustering in the spore, indicating the inactivity of NUDF. In this case, the GFP-BNFA signals were only rarely detected in the vicinity of nuclei, but discrete dots were dispersed throughout the hypha (Fig. 5B). The same cytoplasmic clustering arose in the nudF deletion strain (AGB299), in which definitively no NUDF was present (data not shown). This observation showed that NUDF is required for positioning BNFA at SPBs.

**DISCUSSION**

In this study, we localized fungal NUDC and described its association with NUDF at SPBs and at the cortex. We identified BNFA as a novel NUDF binding protein that is localized at SPBs through its interaction with NUDF. These results lead to the following conclusions. (i) As seen from BiFC and BNFA localization experiments, NUDF is localized at SPBs by means of NUDC and BNFA. (ii) In addition to its cortical localization, fungal NUDC binds NUDF in the cytoplasm and at SPBs at different stages of the cell cycle, showing that A. nidulans might also serve as a model organism for the analysis of mitotic NUDC functions. (iii) There must be a difference between the NUDF subsets at

**FIG. 5.** BNFA is localized to A. nidulans SPBs in a NUDF-dependent manner. (A) Strain AGB297 (nudF ΔbnfA palcA::gfp2-5::bnfA) was grown on MM containing 2% glycerol at room temperature overnight. GFP-BNFA was localized in small amounts throughout the nucleus but accumulated at SPBs during interphase (upper) and mitosis (lower). Arrowheads indicate two adjacent SPBs after duplication at the beginning of mitosis, when GFP-BNFA is not yet localized to the same extent to the new SPB as to the old one. Scale bars, 5 μm. (B) Strain AGB337 (nudF bnfA::gfp2-5) was grown on glucose-containing MM at room temperature overnight and showed the same nuclear dot pattern as that of the SPB-protein γ-tubulin (MIPA). Scale bar, 5 μm. (C) Strain AGB298 (nudF6 palcA::gfp2-5::bnfA) was incubated in MM containing 2% glycerol at 42°C for 8 h and showed widely distributed GFP-BNFA dots along the hypha in the absence of NUDF. Scale bar, 5 μm. DIC, differential interference contrast.
MT plus ends and at SPBs, because neither BNFA nor NUDC was found at MT plus ends, where the amount of NUDF is especially high. (iv) At least at SPBs, the dynein/NUDF complex contains more proteins than assumed until now, as we have shown by copurifying BNFA.

The dynein regulatory complex at SPBs. In *Aspergillus*, NUDF was previously found at the poles of spindles of various lengths, but it was not directly observed there during interphase (35). NUDA was localized there only late during mitosis, for which NUDF was required, among others. Thus, it is not unlikely that small amounts of NUDF also are present there during interphase but could not yet be detected. Using BiFC microscopy, we obtained data indicating that NUDF is present at SPBs at different stages of the cell cycle. In addition, by means of analyzing the presence or absence of the NUDF binding protein BNFA at SPBs in the presence or absence of NUDF, a subset of NUDF could be indirectly localized there during interphase. NUDF’s homolog LIS1, together with dynein, was shown to be necessary for coupling the centrosome to the nucleus in mammalian neurons and *Dictyostelium* (44, 52). LIS1 also was found there throughout the cell cycle, and this localization was not MT dependent in *Dictyostelium*, in contrast to the case for neurons. Therefore, LIS1 was suggested to be an integral centrosome component. The SPB of filamentous fungi differs from the centrosome in that it is embedded in the nuclear envelope. However, this difference does not obviously influence NUDF/LIS1 localization at this MT-organizing center. NUDF was shown to be positioned at spindle poles in the absence of dynein or MTs (35), and it seems likely that NUDGE and CLIPA also are the NUDF anchors at SPBs. Mouse NUDE binds to six centrosomal proteins and is important for MT organization (17), and human Nude1 was described to be necessary for centrosomal LIS1 localization and MT nucleation (23). These results indicate an important recruiting function of NUDE and argue against NUDF/LIS being an SPB/centrosome component.

In our experiments, BNFA was seen only at SPBs and not on MTs or at their plus ends, where there is an abundant supply of NUDF. Therefore, we conclude that BNFA is recruited directly to SPBs by NUDF independently of dynein or MTs. Other anchors for BNFA seem unlikely, since the depletion of NUDF was sufficient to displace BNFA from SPBs. The expression of *bnfa-gfp* and *mipa::mrfp* in a *nudF6* or *nudFA* strain could further confirm the NUDF-dependent localization of BNFA to SPBs. Our observation also makes it unlikely that BNFA is an integral component of the SPB. At present it remains unknown if the NUDF complex is bound to the inner plate or outer plate of SPBs, but BNFA localization within the nucleus indicates binding to both sides of SPBs.

Furthermore, the data presented here indicate that fungal NUDC also is part of the dynein/NUDF complex at SPBs, which suggests that, in *A. nidulans*, NUDC also is involved in MT organization for nuclear migration and spindle formation. In our BiFC study, the NUDF-NUDF interaction was observed in the cytoplasm in addition to SPBs. This localization could be a functional association, or it could be due to the overexpression of the fusion proteins. Overexpression studies of NUDA and NUDE fusions, respectively, showed that in addition to the authentic comets at MT plus ends, immobile cytoplasmic specks also appeared, which were assumed to be artifacts (12). Therefore, we conclude that the SPB localization of NUDC, like that of NUDF, is authentic and due to a specific function at this location, whereas the cytoplasmic localization is an artifact; however, at the moment we cannot rule out that it also is significant.

At present, remains unclear how NUDC is attached to the SPB or the cortex. NUDF might be at least partly responsible for NUDC localization, but the opposite could be true as well. The colocalization of hNUDC with MTs could indicate direct MT binding (42), but it also is possible that NUDC binds to integral SPB components, other dynein complex subunits like NUDE, or additional regulatory proteins. Therefore, a screening for proteins binding to NUDC or to BNFA will be a target of our future research to confirm these localization studies and to clarify the molecular function of the dynein regulatory complex at this MT-organizing center.

Different subsets of NUDF and NUDC. The colocalization of NUDF and BNFA at SPBs, but not MT plus ends, suggests the existence of different subsets of NUDF. Similarly, there might be a difference between the NUDC population at the cortex and the NUDC associated with NUDF at SPBs. At SPBs, important regulatory mechanisms perform functions. Mitotic kinases and phosphatases like NIMA, PLKA, and BIMG are located there in *A. nidulans* and control mitotic events through phosphorylation/dephosphorylation cascades (2, 9, 19). LIS1 is a phosphoprotein (47), and NUDF was shown to be modified (our unpublished data). This raises the possibility that NUDF is phosphorylated at SPBs and therefore associates with specific proteins, like BNFA, at this site but not at others. The described phosphorylation of hNUDC by PLK1 (65) suggests that fungal NUDC also is phosphorylated. hNUDC was shown to bind PLK1, and upon its phosphorylation by PLK1 it associates with kinetochores (41). A similar mechanism also may apply to NUDC in *Aspergillus*. It will be very interesting to investigate if the phosphorylation of these nuclear migration proteins is a means to control the associations with specific factors, which in turn account for the different functions of dynein during the cell cycle.

The dynein/LIS1 complex contains more proteins than previously known. The determination of protein structures and multiple interaction studies lead to a model for LIS1 interaction with Nde11 and dynein proposed by Tarricone et al., in which LIS1 dimerizes via its LisH domain and interacts with a Nudel dimer through the coiled-coil helix (53). In addition, the LisH domains of LIS1 contact the dynein heavy-chain stems, while the WD40 domains reside near the first AAA domain of the heavy-chain heads. In this study, we showed that fungal NUDC can associate with NUDF/LIS1, too, and that it can bind to NUDF’s WD40 domain, which is in agreement with the described structural model, because the WD40 domains seem accessible to additional binding partners. How BNFA is incorporated in this complex is unknown, but it might bind NUDF’s coiled-coil helix via its own C-terminal helix or might also contact the WD40 domain. In our two-hybrid analyses, BNFA did not bind to NUDC, which argues against the latter possibility, but BNFA is able to dimerize and therefore might also form heterodimers with other coiled-coil proteins (our unpublished results).

Here, we showed that there is at least one additional protein that is specifically recruited by NUDF to the dynein complex at
SPBs. The function of BNFA in the dynein/NUDF complex, the existence of homologous genes, and which other proteins might assume its role in other organisms remains to be elucidated. The continuous improvement of genome annotations might complete the search for yet-undiscovered proteins in other organisms. On the other hand, it cannot be excluded that BNFA remains unique to fungi. For another formerly unique SPB-associated protein, SNAD, which affects seption in Aspergillus nidulans (36), analogous proteins now can be found in other fungi. Similarly, the SPB protein APSB seems to be required for MT organization only in fungi (56). Thus, given the slight difference between the SPB of fungi and the centrosome of higher eukaryotes, there could be a difference in dynein function or MT organization at these sites that involves BNFA. It is an interesting goal for future research to decipher BNFA’s molecular function and identify analogous proteins in higher eukaryotes.

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