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 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 NUDC directly interacted with NUDF 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 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 (sterigmatia), uninucleate and green spores are finally released. Without NUDF, mainly anucleate sterigmatia 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 encoding genes reduce the frequency of MT catastrophe and rescue events, respectively, indicating that the intact dynein/NUDF complex promotes plus-end dynamics. This is further supported by the isolation of an α-tubulin mutant, which suppresses the nudA and nudF defects (58). Furthermore, dynein and NUDF regulate the attachment of MT plus ends to the cell cortex; NUDF seems to interact with APSA (for anucleate primary sterigmata A), a cortical landmark protein, so that 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 multiplicity 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 NUDGE (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). Moreover, NUDF is reduced in the nudC mutant, indicating that NUDC acts upstream of NUDF (61). In contrast to the other nud mutants, a nudC deletion in A. nidulans results in the loss of polar growth, aberrant 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. Murein NUDC interacts biochemically with LIS1 and copurifies with the dynein heavy and intermediate chains. Similarly, the colocalization of murein 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 kinetochore 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 nonneural 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% trace 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 trpl his3 ura3 leu2-3,112 tr240 leu2-3,112::ADE2 ura3::LEU2 ura3::LEU2) harboring pBR1840 (URA3 leu2-3,112 tr240 leu2-3,112::ADE2 ura3::LEU2 ura3::LEU2) was transformed with the desired plasmid (31) according to the manufacturer’s instructions.

Transformation procedures. E. coli cells were transformed as described previously (27) or by using electroporation (50) with a Bio-Rad Gene Pulser at 2.5 kV in 0.2-cm cuvettes (Bio-Rad Laboratories GmbH, München, 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::ΔnuF deletion strain was generated for the reintegation 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 Krappmann et al. (32) using the zeo-ApG::zeo deletion cassette for targeted gene replacement. A 10-kb genomic BglII fragment containing nudF was isolated from an A. nidulans genomic library by colony hybridization 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 cloned into BamHI-digested pBluescript (pME2822). This plasmid and the nudF deletion cassette were transformed into E. coli K12 substrains and the plasmid resulting from homologous recombination (pME3231) was rescued from K272. It contained the nudF::zeo::ApG::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 AGB257, yielding strain AGB257 (nudF::ΔnuF::zoe::ApG::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-fluoroorotic acid (5-FOA) medium according to Krappmann et al. (32), resulting in strain AGB294.

A TAP-tagged version of nudF was reintegrated into the nudF locus of AGB257. An MluI 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 MluI site (stap+; 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 AGB294 (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.
Plasmid and strand constructions for bnfA deletion. The bnfA knockout strain was generated by using the zeo-Afp2r-zeo deletion cassette. A 986-bp 5′ UTR and a 963-bp 3′ UTR fragment were PCR amplified and cloned into the EcoRV and HpaI restriction sites of pME2409, respectively (OLKH241, 5′-ATG TGG ATT CTC TAT TGG ACC CGT AG-3′; OLKH242, 5′-GGA GCT GGC TGG AGA AGA CCA TTA GAC TAC AC-3′; and OLKH244, 5′-AAC CTG TTT CAT TGG ACC CGT AG-3′), resulting in plasmid pME2323. The linearized plasmid was transformed into the ΔnudA 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 ΔnudA strains was unsuccessful, the ΔnufA:zeo deletion also was generated in the AGB152 strain background (AGB296), and the last one was used for localization experiments.

Plasmid and strand constructions for localization experiments. NUDC-green fluorescent protein (NUDC-GFP) was localized in an A. nidulans nudC3 strain after the transformation of pME823 harboring a nudC::zeo fusion into A779 (AGB241). pME243 was created by cloning a KpnI fragment of nudC into plasmid pME2323. The resulting plasmid, pME3238, was transformed into the AGB152 strain background (AGB296), and the last one was used for cloning.

<table>
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<th>Strain</th>
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This study

For the colocalization of NUDF and NUDC, the encoding genes were fused together with pME3173 for the expression of the nuclear marker.

PCR product comprising a mipa::mrfp::gpdA::nalR cassette (AGB335). For this fusion PCR, a template plasmid was generated: a (GA) 5′ linker, the natR expression module were amplified with primers OLKH301 (5′-GGA ATT CGG AGC TGG TGC AGC CCG TGC CCG CTC CTC CGA GGA GCT C-3′), OLKH302 (5′-ACC GCT CAT TGG AGA GAG CAG TGG CGG TGC CTC TCT CGA GGA GCT C-3′), and OLKH303 (5′-CCA TCT CCG CGG CTA AGC GGC AGC AGC ACC TCT CGA GGA GCT C-3′). This module was generated by fusion PCR (OLKH304, 5′-AAC CTG TTT CAT TGG ACC CGT AG-3′; OLKH305, 5′-GGA GCT GGT GCA GGC ATG CTG TCC TCT CTA TGG ACC AG-3′). For this study.

Downloaded from http://ec.asm.org/ on July 7, 2015 by Maynooth University.
A Western hybridization with a rabbit anti-calmodulin binding peptide antibody (Upstate/Millipore GmbH, Schwabach, Germany) was performed on samples taken during the purification procedure, which were transferred to a nitrocellulose membrane by being electrobotted after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were detected by the enhanced chemiluminescence method (54).

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 at 546 nm of 0.2 with SC medium, and 10 μl of the dilution was spotted onto SC-4 plates containing either 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 μM leucine for subsequent β-galactosidase filter assays.

Microscopy. Five hundred microliters of the appropriate medium was put onto coverslips, which were placed in petri dishes and incubated with 4 × 10⁶ spores.

For growth at 42°C, coverslips were placed into 6-well plates with 5 ml of medium
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 photographs were taken using a Xilix MicroImager digital camera (Xilix Technologies Corp., Richmond, Canada) and the Openlab software (Improvision, Coventry, United Kingdom).

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, 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). 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/tgd/c2k1/afu1/), the Broad Institute (www.broad.mit.edu/annotation/fungi/afu1/), the Center for Integrated Fungal Research (www.aspergillusflavus.org), and the COILS server (www.ch.embnet.org/software/COILS_form.html). Protein alignments were carried out with NPSA (npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_clustalw.html).

RESULTS

NUDC 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 NUDE (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-NUDF 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.

![Image](http://ec.asm.org/ on July 7, 2015 by Maynooth University)
NUDF presumably contains an N-terminal LisH dimerization motif followed by a coiled-coil helix and a large WD40 domain (a β-sheet propeller) (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 and lacZ reporter gene expression (EGY48 harboring pRB1840, pME3246, and pME3237 and EGY48 harboring pRB1840, pME3246, and pME3243). NUDF binding to the coiled coil of NUDC (EGY48 harboring pRB1840, pME2938, and pME2939) was used as the positive control (PC). NUDF and the empty prey vector (EGY48 harboring pRB1840, pME2938, and pJG4-5) were used as the negative control (NC). BiFC. Strain AGB303, expressing the nudF::neyfp and nudC::ceyfp fusions and the nuclear marker mrfp::h2A, was cultivated in MM at 42°C for 8 h. The colocalization of NUDF and NUDC was shown through the fluorescence emission, which could be generated only by the colocalization of the N- and C-terminal halves of eYFP (examples are indicated by arrowheads). Scale bars, 5 μm. BiFC. Strain AGB335, expressing the BiFC constructs and the SPB marker mipA::mrfp, was grown in MM at 42°C for 8 h. Scale bars, 5 μm. DIC, differential interference contrast.

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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 niiA/niaD promoter by growth on nitrate-containing medium and partially complemented the temperature-sensitive phenotypes when expressed in the nudC3 and nudF6 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-nEYFP 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 /H9253-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).

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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+/H11001 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 ΔnudF 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 specifically 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*, and *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 presumable 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 NUDE 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 *ΔkuA* 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 *ΔbnfA* 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::
gfp2-3 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 in-
teraction, 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 asso-
ciation 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 possi-
bilities. 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 local-
ized 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 through-
out the hyphae (Fig. 5B). The same cytoplasmic clustering
arose in the nudF deletion strain (AGB299), in which defini-
tively no NUDF was present (data not shown). This observa-
tion 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 identi-
ified 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 not only
during mitosis but also during interphase, although its amount
there obviously is much smaller than that at the plus-end res-
ervoir. (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
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 NUDA 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 nudFΔ 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, it 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 Nde1 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
SBPs. 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 concluded that BNFA remains unique to fungi. For another formerly unique SPB-associated protein, SNAD, which affects septation in \textit{A. nidulans} (36), analogous proteins now can be found in other fungi. Similarly, the SBP 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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