Ras1-Induced Hyphal Development in *Candida albicans* Requires the Formin Bni1

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Formins are downstream effector proteins of Rho-type GTPases and are involved in the organization of the actin cytoskeleton and actin cable assembly at sites of polarized cell growth. Here we show using in vivo time-lapse microscopy that deletion of the *Candida albicans* formin homolog BNI1 results in polarity defects during yeast growth and hyphal stages. Deletion of the second *C. albicans* formin, BNR1, resulted in elongated yeast cells with cell separation defects but did not interfere with the ability of bni1 cells to initiate and maintain polarized hyphal growth. Yeast bni1 cells were swollen, showed an increased random budding pattern, and had a severe defect in cytokinesis, with enlarged bud necks. Induction of hyphal development in bni1 cells resulted in germ tube formation but was halted at the step of polarity maintenance. Bni1-green fluorescent protein is found persistently at the hyphal tip and colocalizes with a structure resembling the Spitzenkörper of true filamentous fungi. Introduction of constitutively active rasG12V in the bni1 strain or addition of cyclic AMP to the growth medium did not bypass bni1 hyphal growth defects. Similarly, these agents were not able to suppress hyphal growth defects in the wall mutant which is lacking the Wiskott-Aldrich syndrome protein (WASP) homolog. These results suggest that the maintenance of polarized hyphal growth in *C. albicans* requires coordinated regulation of two actin cytoskeletal pathways, including formin-mediated secretion and WASP-dependent endocytosis.

Cell polarity establishment and maintenance of polarized secretion are essential for morphogenesis and development (10). Cell polarization is required in neuronal cells to establish a growth cone that maintains a polarized growth direction in response to extracellular stimuli (9, 20). Cell polarization is also required for epithelium formation and in migrating cells. Similarly, in plant cells, establishment and maintenance of cell polarity are used during root hair or pollen tube growth (31). Both actin and microtubule cytoskeletons play important roles in maintaining cell polarization and in providing cellular tracks for vesicle delivery. This requires complex processes of spatial and temporal coordination of protein localization and activation at sites of polarized growth (32).

The actin cytoskeleton is involved in three basic structures: actin patches, actin cables, and the cytokinetic ring at sites of cell cleavage in animal and fungal cells. Actin patches are found at sites of endocytosis, actin cables provide tracks for vesicle delivery, and dynamic constriction of the actin ring is required for cytokinesis (32).

Rho-type GTPases, such as Cdc42, are known regulators of the actin cytoskeleton in that they activate downstream effector proteins (11). Two major classes of conserved effector protein families are the Wiskott-Aldrich syndrome proteins (WASPs) and the formins. WASP-like proteins are involved in endocytosis and play a role in Arp2/3-dependent actin assembly (7, 26, 43). The *C. albicans* WASP homolog, Wal1, was shown to be required for endocytosis and vacuolar morphology as well as polarized hyphal growth (40). Formins represent the conserved family of Diaphanous-related proteins that control the assembly of actin cables (33, 34). Formins assemble linear actin cables in an Arp2/3 complex-independent manner (12, 13, 33, 34).

The sole *Aspergillus nidulans* formin, SEPA, is so far the only formin analyzed in filamentous fungi. SEPA localizes to sites of polarized growth both at the hyphal tip and at septal sites. The tip localization resembles the position of a structure termed the Spitzenkörper (17). In *Saccharomyces cerevisiae* the formin Bni1 colocalizes with Spa2, Bud6, and Pea2 at sites of polarized growth and forms a complex termed the polarisome (15, 36). In *C. albicans*, SPA2 has been analyzed recently and was found to localize to the tips of growing hyphae. Consistently, deletion of *SPA2* resulted in polarity and hyphal growth defects (44).

In this study, we exploit the human fungal pathogen *Candida albicans* as a model to understand the role and contribution of formins in the regulation of polarized morphogenesis. In *C. albicans*, the yeast-to-hyphal transition contributes to its virulence and allows the penetration of epithelia and the evasion of the host cellular immune response (6). The genetic basis of morphogenetic switching in *C. albicans* relies on the activation of the Ras1-GTPase by extracellular signals which induce two downstream signal cascades: a mitogen-activated protein (MAP) kinase pathway and the cyclic AMP (cAMP) pathway. Both activate transcriptional regulators, Cph1 and Efg1, respectively, that induce hypha-specific gene expression (4). Deletions of *CPH1* and *EFG1* or a single deletion of both *RAS1* alleles yields viable mutant strains that are nonfilamentous under most conditions and avirulent in animal models (14, 25).

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On the other hand, constitutive activation of \textit{RAS1}, using a \textit{ras1}^{G13V} allele, enhances filamentous growth (15). In this study, we investigate the role of the \textit{C. albicans} \textit{formins} as part of a potential Spitzenkörper complex and present a model including the actin cytoskeleton machinery in the regulatory network that establishes hyphal morphogenesis in \textit{C. albicans}.

**MATERIALS AND METHODS**

**Strains and media.** \textit{C. albicans} strains used in this study are listed in Table 1. Media and the lithium acetate transformation procedure were used as described previously (39, 40).

**Targeting of \textit{C. albicans} genes.** The \textit{C. albicans} homologs of the \textit{Saccharomyces cerevisiae} \textit{formins} \textit{BN11} and \textit{BN12} were identified in the \textit{C. albicans} genomic sequence (http://www-sequen.stanford.edu/group/candida). Deletions of the complete open reading frames (ORFs) of both alleles of \textit{C. albicans} \textit{BN11} (CABN11) or \textit{BN12} were performed by PCR-generated \textit{URA3} and \textit{HIS1} disruption cassettes containing 100 bp of a target homology region at both ends of the cassettes as described previously (18). Similarly, regulatable expression of \textit{BN11} under the control of the \textit{CaMAL2} promoter was achieved by using amplified \textit{HIS1}-\textit{MAL2p} cassettes in the transformation of a \textit{BN11}:\textit{bn11} strain so that the endogenous promoter of the only remaining copy of \textit{BN11} is replaced with the \textit{MAL2} promoter. To reconstruct strains generating new marker combinations in the absence of a sexual cycle, a homozygous strain (in this case the \textit{bn11}:\textit{bn11}:\textit{bn11}:\textit{ADE2}) was used and transformed with PCR-amplified cassettes derived from the \textit{pFA-ARG4} plasmid. Upon double selection for histidine and arginine prototrophy, transformants whose \textit{HIS1} marker was replaced with the \textit{ARG4} marker gene were selected. Either this method or use of a suitable heterozygous strain can result in generating the desired marker combinations. To increase the arsenal of available marker combinations, we generated a \textit{pFA-SAT1} plasmid which contains the \textit{SAT1} gene (kindly provided by Joachim Morschhäuser). Its use will be described in detail in an upcoming publication on \textit{pFA} modules.

In order to fuse \textit{BN11} with green fluorescent protein (GFP), a fusion cassette was cloned. To this end, a PCR fragment was amplified from genomic DNA which contains the 3’ end of \textit{BN11}. The in vivo recombination machinery of \textit{Saccharomyces cerevisiae} was used to fuse the end of the \textit{BN11} ORF with GFP. To this end, a GFP-\textit{URA3} PCR cassette was amplified from the standard set of modules. This cassette contains \textit{C. albicans} \textit{URA3}, which is also functional in \textit{S. cerevisiae}. Gene targeting in \textit{S. cerevisiae} requires shorter homology regions that flank the target locus (45 bp were used in our case). This detour via \textit{S. cerevisiae} was used to generate a cassette and to verify the correct in-frame fusion after plasmid recovery from yeast and amplification in \textit{Escherichia coli} by sequencing. This cassette was excised from the plasmid backbone and used for the transformation of \textit{BWP17}, yielding strain GC59.

The constitutively active \textit{ras1}^{G13V} allele was used as described previously, which resulted in targeted integration of the \textit{CaMAL2} promoter-driven alleles at the \textit{C. albicans} \textit{ADE2} locus (14).

Primers used for the construction of cassettes and the verification of deletions are listed in Table 2 or were described previously (40). Disruptions were verified by PCR of whole yeast cells.

**Hyphal induction of \textit{C. albicans}**. Different protocols were used to induce hyphal formation in \textit{C. albicans} strains at 37°C. On solid plates, hyphal induction was done either on Spider medium (24) or on medium containing 10% serum (calf serum; Sigma). Plates were incubated for 4 to 7 days prior to photography. In liquid culture, the addition of 10 mM CAMP was used besides serum to induce filament formation.

**Staining procedures.** Chitin staining was done by directly adding 1 μl calcofluor (1 mg/ml) to a 100-μl cell suspension, followed by an incubation of 15 min at room temperature and a subsequent washing step to optimize the signal-to-noise ratio. Vascular staining of overnight cultures was done using the lipophilic dye FM4-64 (0.2 μg/ml). Cells were stained for 30 min at 30°C prior to photography. To monitor the uptake of FM4-64 by in vivo time-lapse microscopy, cells in the exponential growth phase were transferred to deep-well microscopy slides containing the dye in the culture medium. Imaging of the Spitzenkörper-like structure was done by staining cells induced for germ tube formation with FM4-64 immediately prior to microscopy. Staining of vacuoles in germ tubes was done by adding FM4-64 to the culture during hyphal induction, which was carried out for 3 h at 37°C in the dark in minimal medium containing 10% serum. Rhodamine-phalloidin staining of the actin cytoskeleton was done as described previously (29).

**Time-lapse microscopy.** Strains were grown to exponential phase, harvested, washed, and resuspended in sterile water. Small aliquots of cells (1.5 μl) were applied to microscopy slides with deep wells as described previously (38). Temperature control was achieved via a heat stage mounted on the microscope table. Microscopy was performed on an automated Zeiss Axiosplan II imaging microscope. Image acquisition using Metamorph software (Universal Imaging Corporation) and movie processing were done as described previously (40). Movies are posted at the corresponding author’s homepage at http://pinguin.biologie.uni-jena.de/phytopathologie/pathogenepilze/index.html.

Sequence data for \textit{Candida albicans} was obtained from the Stanford Genome Technology Center website at http://www-sequence.stanford.edu/group/candida.

**RESULTS**

**Functional analysis of \textit{C. albicans} \textit{formins}.** \textit{Candida} \textit{formins} were identified in the \textit{C. albicans} genome sequence using the \textit{Saccharomyces cerevisiae} \textit{Bni1} protein. Two forms were found: \textit{CaBN11} and \textit{CaBN12} of 1,732 and 1,485 amino acids in length, respectively. \textit{CaBN11} is annotated as orf19.4927 and \textit{CaBN12} as orf19.7537. Both forms correspond in their protein structure to \textit{A. nidulans} SEPA and other \textit{formins} that they possess an \textit{N}-terminal \textit{G} protein binding domain, followed by a formin homology 3 (FH3) domain and a C-terminal FH1-FH2-Diaphanous autoinhibitory domain (DAD) involved in actin filament assembly and regulation of \textit{formin} activity by

| Strain* | Genotype | Reference |
|---------|----------|-----------|
| SC5314  | \textit{Candida albicans} wild type | 16 |
| BWP17   | \textit{ura3}:\textit{linm34}:\textit{ura3}:\textit{linm34} \textit{arg4}:\textit{hisG}\textit{arg4}:\textit{hisG} \textit{his1}:\textit{hisG} \textit{his1}:\textit{hisG} | 42 |
| CAT4    | \textit{WAL1}:\textit{wal1}:\textit{HISI} | 40 |
| CAT6    | \textit{wal1}:\textit{HIS1}:\textit{wal1}:\textit{URA3} | 40 |
| CAT37   | \textit{wal1}:\textit{HIS1}:\textit{wal1}:\textit{SAT1} | This study |
| CAT40   | \textit{wal1}:\textit{HIS1}:\textit{wal1}:\textit{SAT1}:\textit{ADE2}:\textit{ade2}:\textit{MAL2}:\textit{rast}\textit{G13V}:\textit{URA3} | This study |
| GC11    | \textit{BN11}:\textit{bn11}:\textit{URA3} | This study |
| GC13    | \textit{BN11}:\textit{bn11}:\textit{URA3} | This study |
| GC14    | \textit{bn11}:\textit{URA3}:\textit{bn11}:\textit{HIS1} | This study |
| GC19    | \textit{bn11}:\textit{URA3}:\textit{bn11}:\textit{HIS1} | This study |
| GC33    | \textit{MAL2p-\textit{BN11-\textit{HIS1-\textit{bn11-\textit{URA3}}} | This study |
| GC40    | \textit{bn11}:\textit{HIS1}:\textit{bn11}:\textit{ARG4} | This study |
| GC42    | \textit{ADE2}:\textit{ade2}:\textit{MAL2p-\textit{rast}\textit{G13V}:\textit{URA3} | This study |
| GC46    | \textit{bn11}:\textit{HIS1-\textit{bn11-\textit{ARG4-\textit{ADE2-ade2}:\textit{MAL2p-\textit{rast}\textit{G13V}:\textit{URA3} | This study |
| GC59    | \textit{BN11}:\textit{BN11-\textit{GFP-\textit{URA3} | This study |

* All strains whose names begin with GC and CAT are derivatives of BWP17 but have the indicated genotypic alterations.
autoinhibition (30). The highest sequence identity of more than 50% between C. albicans Bnl1 and the S. cerevisiae Bni1 is found in the C termini, including the FH2 domains, while the N termini are less well conserved and show less than 30% identity on the amino acids level. The presence of conserved residues of the G protein binding domain and DAD indicates that regulatory mechanisms similar to those that have been described for other systems may apply in C. albicans, particularly the direct binding to a Rho-type GTPase.

To investigate the role of both C. albicans formins during yeast and hyphal growth, we constructed mutant strains in which the complete ORFs of both alleles of BNI1 or BNR1 were sequentially deleted using PCR-based gene targeting methods (18). Homozygous mutant strains were generated from independent heterozygous strains for each formin and were phenotypically identical, indicating that correct gene targeting had occurred, as was verified by colony PCR. The absence of the formin ORFs in the homozygous but not in the heterozygous mutant strains was shown using internal primers. Additionally, to provide further proof that the observed phenotypes described below were solely due to the disruption of BNI1, a heterozygous BNI1/bni1 mutant strain was used to place the only remaining copy of the target gene under the control of the regulatable CaMAL2 promoter. This generated strains with wild-type-like phenotypes, including the ability to filament under permissive conditions during growth on maltose, while a shutdown of the MAL2 promoter during growth on glucose restored mutant phenotypes (not shown).

### Polarized growth defects of formin mutants during the yeast growth phase

Cell cycle lengths of the wild type and the bni1 and bnr1 strains were determined using growth curves generated with cultures grown in liquid rich medium at 30°C and using in vivo time-lapse microscopy recordings calculating as the average cell cycle duration the time required between two consecutive bud emergence events. This revealed prolonged cell cycle durations of about 20 min for both formin mutants compared to that for the wild type (Table 3). Wild-type cells are characteristically ellipsoidal and show a length-versus-width ratio of approximately 1.3. During growth on solid media, cytokinesis in the wild type is most obvious by a rotational movement of mother and daughter cells out of the mother-bud axis (Fig. 1A). Heterozygous mutants with either BNI1/bni1 or BNR1/bnr1 were phenotypically like the wild type, suggesting the absence of gene dosage effects.

Bud emergence in bni1 cells was not inhibited. However, newly formed buds showed drastic defects in polarized growth. This resulted in the formation of swollen and rounded yeast cells with a length/width index of 1.1, resembling mutant wal1 cells bearing a deletion of the WASP homolog WAL1. Strong defects were observed at the bud neck, which was broadened and revealed enlarged septa (Table 3). Separation of mother and daughter cells did not take place as readily as in the wild

| Primer       | Sequencea |
|--------------|-----------|
| 392 XFP      | GAGTGCCATGCCGGAAGGTATAGT |
| 599 U3       | GAGGTTGGAGTTAGTGAAGATG |
| 600 U2       | GTTGTGCAATCTAAGGCAATAC |
| 601 H2       | CCAAGAATGCGCTCCCTACCAAG |
| 602 H3       | GAGCAGATTGAGAAAGTGCTGGAC |
| 797 S1-BNI1  | ATCACAACATCCCCATCGACATAC |
| 798 S2-BNI1  | CTTAACATGAACTCATGATGATAATAGT |
| 799 G1-BNI1  | CTCTCTGGGGAGGACACAGCG |
| 800 G4-BNI1  | CAGGTTCGCAAATGGAATCTCC |
| 801 G4-BNI1-MAL2p | GCCCTCTTGCCTGATG |
| 802 G1-BNI1-FP | GGCGCTGAAAGGATCGT |
| 804 S2-BNI1-MAL2p | GGTGAGTTCGATGGTGATGATAGT |
| 805 S1-BNI1 | GTTGTTTTTTTTTTTTCCAACCGACATCTAAATATACATAGCAGTTTCAATTAATTACCATATTC |
| 806 S2-BNI1 | AAAATGGAGAAAAAAGAAAAAAAAGAAAAAAGAAAAAGAAAAAAATAGTTGTTCTTTTATTTAGGAAGAGACATACA |
| 811 G1-BNI1 | GTAAGCACCCAGGACTTCTGTC |
| 812 G4-BNI1 | GGAATTTTCTACCAACAGAGC |
| 1088 II-BNI1 | GGAATACTAAGAAACACAGACCTTG |
| 1089 II-BNI1 | CTTCTGGAACAGCGCAACAC |
| 1090 II-BNR1 | GAGAGATAGTTCCAGGAACACAG |
| 1091 II-BNR1 | GCCACATGGCTTGCAGCTAC |
| 1203 CaBNI1 | CGGCGGTAGCACCACTAACATATGCTCACC |
| 1204 CaBNI1 | TGCTCTAGACAGCAGACTTACATTTATTAATGGAGGAAGATGAA |
| 1242 ADE2-down | GGTGCTGATGATGTTAGGAAGCAGC |
| 1243 ADE2-up | CAGAGATGGTGGCTGCGT |
| 1244 RAS1-down | GGAAGAACAGTTGTTATCAGAGG |
| 1268 S1-BNI1-GFP | GTTCAAAATGAGCTTTGTAGTGAAGTGGCTAAGAATAAAGAATAGTGAGGAGGACACAGTTGTCAGGCTGCAAGGAGGAAGCATTCAGTCTTTATGATGTTAAATAG |
| 1269 S2-BNI1-GFP | CTCTGACTTATGATGATGTTAGGAAGTGGCTAAGAATAAAGAATAGTGAGGAGGACACAGTTGTCAGGCTGCAAGGAGGAAGCATTCAGTCTTTATGATGTTAAATAG |

a Uppercase sequences correspond to C. albicans genomic DNA. Lowercase sequences correspond to 3’-terminal annealing regions for the amplification of transformation cassettes. Bold letters indicate restriction sites used for cloning. All sequences are written from 5’ to 3’.
type. Instead mother and daughter cells were separated by pushing forces generated by newly formed buds during growth on solid media as observed during in vivo time-lapse recordings (Fig. 1B).

Characteristically, \textit{bnr1} cells were more elongated than the wild type, which resulted in a length/width ratio of 1.7. Cell separation defects of \textit{bnr1} cells were not as drastic as those seen in \textit{bni1} cells but still resulted in mother and daughter cells

![Image](http://pinguin.biologie.uni-jena.de/phytopathologie/pathogene/pilze/index.html), a characteristic change of cell axes after cytokinesis can be observed (previous mother-bud axes are indicated as white, dotted lines and newly established axes as black, dashed lines in panels A to C). This results in lateral movement of cells such that wild-type colonies form a single cell layer (A). In the \textit{bni1} mutant, growth was irregular and cells were dispatched in three dimensions. A shift of mother-bud axes occurs but often only due to mechanical forces generated by new buds. The arrows denote enlarged septal sites (B). In the \textit{bnr1} mutant, growth axes are kept over several cell cycles, resulting in a linear array of mother and daughter cells (C). Cell shape and budding pattern of the indicated strains were analyzed after staining the cells with calcofluor (D). wt, wild type. Scale bar, 5 \(\mu\)m.

**Table 3. Yeast phase growth characteristics**

| Parameter                      | Wild-type SC5314 | GC14 (\textit{bni1}/\textit{bni1}) | GC39 (\textit{bnr1}/\textit{bnr1}) |
|-------------------------------|------------------|-----------------------------------|-----------------------------------|
| Cell cycle duration (min)     | 71 ± 7           | 92 ± 15                           | 99 ± 14                           |
| Cell length (\(\mu\)m) \((n = 500)\) | 6.0 ± 0.5        | 6.4 ± 0.6                         | 6.7 ± 0.5                         |
| Cell width (\(\mu\)m) \((n = 500)\) | 4.6 ± 0.7        | 5.7 ± 0.4                         | 3.9 ± 0.4                         |
| Bud scar diameter (\(\mu\)m) \((n = 200)\) | 1.5 ± 0.3        | 2.4 ± 0.9                         | 1.5 ± 0.4                         |

*Values are means ± standard deviations.*

**Fig. 1.** Growth defects of \textit{C. albicans} formin mutants during yeast growth. Growth of the wild type and formin mutant strains was monitored using time-lapse microscopy over several hours (timescale is in hours:minutes) (A-C). In the movie of the wild type at [http://pinguin.biologie.uni-jena.de/phytopathologie/pathogene/pilze/index.html](http://pinguin.biologie.uni-jena.de/phytopathologie/pathogene/pilze/index.html), a characteristic change of cell axes after cytokinesis can be observed (previous mother-bud axes are indicated as white, dotted lines and newly established axes as black, dashed lines in panels A to C). This results in lateral movement of cells such that wild-type colonies form a single cell layer (A). In the \textit{bni1} mutant, growth was irregular and cells were dispatched in three dimensions. A shift of mother-bud axes occurs but often only due to mechanical forces generated by new buds. The arrows denote enlarged septal sites (B). In the \textit{bnr1} mutant, growth axes are kept over several cell cycles, resulting in a linear array of mother and daughter cells (C). Cell shape and budding pattern of the indicated strains were analyzed after staining the cells with calcofluor (D). wt, wild type. Scale bar, 5 \(\mu\)m.
that were moved apart by colonial growth rather than by the cytokinesis of mother and daughter cells (Fig. 1C).

To determine if the swollen morphology of bni1 cells resulted also in an altered bud site selection pattern, cells were stained with calcofluor to visualize chitin-rich septal rings (Fig. 1D). In the wild type and in bnr1 cells grown at 30°C, a bipolar budding pattern was observed. In contrast, bni1 cells showed increased random budding resembling that of spa2 and wall1 cells described in previous studies (Fig. 1D) (40, 44).

C. albicans formin mutants do not show defects in endocytosis. Since we found defects in endocytosis in wall1 mutant cells in a previous study, we wanted to determine any deficiencies in vacuolar morphology and transport to the vacuole in formin mutant strains. To this end, we analyzed the uptake of the lipophilic dye FM4-64 by in vivo fluorescence time-lapse microscopy. This revealed no vacuolar phenotypes in bni1 and bnr1 yeast cells compared to the drastic defects in the wall1 mutant, suggesting that formins are not involved in endocytosis.
or in determining the vacuolar morphology of yeast cells (Fig. 2A; movies M4 to M6 at http://pinguin.biologie.uni-jena.de/phytopathologie/pathogenepilze/index.html). For comparison of FM4-64 uptake under inducing conditions, we induced yeast cells of the wild type and the formin and wal1 mutant strains to form germ tubes in the presence of serum at 37°C and simultaneously stained these cells with FM4-64. In the wild type, dye uptake resulted in staining of the apical compartment. Smaller vesicles in apical regions—sometimes at the hyphal apex—were followed by larger vacuoles in subapical compartments. A similar distribution of endosomes and vacuoles was found in the bni1 and bnr1 mutant strains (Fig. 2B). In contrast, defects in endocytosis were observed in induced wal1 cells. Here, we found that dye uptake revealed an accumulation of vesicles at the tip of the germ tubes, indicating defects in the transport or fusion of vesicles.

The C. albicans formin Bni1 is required for the maintenance of polarized hyphal growth. To analyze growth defects during the hyphal growth phase in formin mutants, germ tube formation was initiated under different inducing conditions. First, we used in vivo time-lapse microscopy and followed germ tube induction and polarized hyphal growth of the wild type and the formin mutant strains (Fig. 3; movies M7 to M9). The wild type and also the bnr1 strain responded to serum as a hypha-inducing cue, with vigorous filamentation and the development of mycelia after several hours of growth. In contrast, bni1 cells initiated germ tube formation but generated swollen germ tubes with widened diameters. These hyphal tubes were not able to maintain polarized cell growth, did not generate fast-growing hyphal filaments and lateral branches, and were thus not able to develop into mycelia. Frequently, constrictions were observed at septal sites in otherwise enlarged and swollen cells.

Growth on solid-medium plates containing serum or on Spider plates revealed abundant filamentation at the edges of wild-type and bnr1 colonies, while bni1 colonies showed smooth edges generated by yeast cell growth, supporting our time-lapse data and indicating that even after prolonged incubation, filamentation is crippled in bni1 strains (Fig. 4).

Analysis of the actin cytoskeleton in formin mutants. Distribution of actin patches and cables was analyzed to determine if the morphological defects observed can be attributed to a disorganization of the actin cytoskeleton (Fig. 5). During wild-type yeast growth, cortical actin patches accumulate in the growing bud and actin cables are found oriented at the mother-bud axis. This was also observed in the formin mutant strains, although some partial delocalization of actin patches in bni1 cells was found, resulting in patch positioning in the mother instead of a total accumulation of patches in the bud. This partial delocalization besides the prolonged cell cycle and the cytokinesis defect may explain the cell shape defects in bni1 cells. In bnr1 mutant cells, elongated buds showed clustering of...
cortical actin patches at the broadened tip region (Fig. 5A). During germ tube induction, the actin cytoskeleton in the wild type is strongly polarized towards the hyphal tip as in true filamentous fungi. Such an organization of the actin cytoskeleton was also found in the formin mutants (Fig. 5B). This was expected in \textit{bnr1} hyphae since hyphal development is not blocked in this mutant. However, in the \textit{bni1} germ tubes, given their morphological defects, it was rather unexpected to find such a clearly polarized arrangement of the actin cytoskeleton. Since cortical actin patches are sites of endocytosis, Bni1 may not be involved in the correct positioning of patches.

Subcellular localization of Bni1-GFP during hyphal growth. In \textit{S. cerevisiae}, Bni1 forms a polarisome complex with Spa2 and Bud6 (36). Since the Spa2 homolog in \textit{C. albicans} was localized to the tips of growing hyphae, we wanted to determine the subcellular localization of Bni1. To this end, a C-terminal fusion of GFP to Bni1 was employed in a heterozygous background (Fig. 6A). Throughout polarized hyphal growth, Bni1-GFP was found to localize to the hyphal tip as a punctate spot. However, the label was too weak to generate prolonged time-lapse series. Interestingly, during our studies on the uptake of FM4-64, we found that very short incubation periods of germ tubes or hyphae with the dye resulted in a spot-like staining at the hyphal tip. Staining of Bni1-GFP-expressing hyphae with FM4-64 showed that both spots colocalize (Fig. 6A). This suggests that in \textit{C. albicans} hyphae, a structure is present that resembles the Spitzenkörper of true filamentous fungi, which on the molecular level can be described by the localization of Bni1 (17, 19). Such a Spitzenkörper was not found in yeast-like cells and thus represents a cellular marker that distinguishes the different cell types in \textit{C. albicans}. Bni1-GFP was found to simultaneously localize to the hyphal tip as well as to a site of future septation in \textit{C. albicans} (Fig. 6B).

Next, we determined if the Spitzenkörper is also present in \textit{bni1} germ tubes. In contrast to the wild type, which possesses a focused, point-like Spitzenkörper, in the \textit{bni1} germ tubes, the Spitzenkörper was much broader and enlarged, which indicates that the subcellular structure of the Spitzenkörper correlates with the hyphal diameter (Fig. 6C).

\textit{ras1}^{G13V} does not suppress the \textit{bni1} and \textit{wal1} phenotypes. Maintenance of polarized hyphal growth, but not the initiation of germ tube formation, was found to be blocked in both the \textit{bni1} and \textit{wal1} germ tubes. Since morphogenetic switching depends on the activation of the Ras1-GTPase, we wanted to determine if constitutive activation of Ras1 could bypass the \textit{bni1} or \textit{wal1} defects in polarized hyphal growth (Fig. 7A). To this end, the homozygous \textit{bni1::HIS1/bni1::URA3} strain was used and its \textit{URA3} marker was exchanged for the \textit{ARG4} marker using a PCR-based approach. We then used an integrative cassette containing the \textit{URA3} selectable marker gene, which targets the \textit{ras1}^{G13V} allele under the control of the \textit{MAL2} promoter to the \textit{ADE2} locus and placed this cassette in the BWP17, \textit{bni1}, and \textit{wal1} backgrounds (14). Under inducing conditions (37°C and 10% serum) in the presence of maltose as the carbon source, wild-type and BWP17 strains (with or without the activated \textit{ras1}^{G13V} allele) showed profuse filaments both in liquid culture and on solid media (Fig. 7A). In contrast, \textit{bni1} germ tubes were phenotypically identical to those of strains harboring the activated \textit{ras1}^{G13V} allele. Moreover, the

FIG. 4. Mycelial growth defects of the \textit{bnl1} mutant. The indicated strains were grown for 4 days at 37°C on plates containing either 10% serum or Spider medium prior to photography. Representative images of the edges of colony sectors which demonstrate the mycelial growth defects of the \textit{bnl1} mutant are shown. wt, wild type.
constitutive ras1\textsuperscript{G13V} allele in the bni1 mutant background did not result in filamentation on solid media (insets in Fig. 7A). This indicates that ras1\textsuperscript{G13V}, which in the wild-type background induces filamentation even at 30°C in complete medium (not shown), is not able to bypass the bni1 defect. Similarly, the phenotype of the wal1 mutant, which was previously shown to be defective in maintaining filamentous growth, was also not suppressed by the constitutive ras1\textsuperscript{G13V} allele. This suggests that both pathways, including polarisome function and secretion, as well as endocytosis, are required for a stable hyphal growth phase in C. albicans.

Furthermore, as with the expression of the ras1\textsuperscript{G13V} allele, addition of cAMP to the culture medium did not generate mycelial growth in either bni1 or wal1 cells but readily resulted in filamentation in the wild-type strain (Fig. 7B).

**DISCUSSION**

*C. albicans* can adopt a variety of morphologies; however, there are growth conditions ensuring stable yeast and filamentous-growth phases. The ability to induce hyphal growth in *Candida* allows its use as a model system to understand the
underlying molecular mechanisms. One of the interesting questions is how a conserved basic protein network can be regulated differentially to achieve dimorphic growth in contrast to the continuous polarized growth of true filamentous fungi, for example, *Neurospora crassa*, *Aspergillus nidulans*, and *Ashbya gossypii* (5, 27, 41).

In true filamentous fungi, an apical body can be distinguished, that is, it is phase dark and forms at growing hyphal tips, and was found to be responsible for the growth direction of hyphal tips (1, 17, 19). This apical body, or Spitzenkörper, serves as the vesicle supply center in support of hyphal growth (2, 17). This Spitzenkörper can be stained by the lipophilic dye FM4-64, which stains endocytic vesicles (19). We have previously used this dye in the analysis of endocytosis in *C. albicans*. Deletion of the Wiskott-Aldrich syndrome protein *C. albicans* homolog *WAL1* resulted in mutant strains that showed defects in the maintenance of polarized hyphal growth and were unable to form mycelia (40). Staining of yeast cells with FM4-64 did not reveal a prominent accumulation of vesicles but rather resulted in the delivery of the dye to and the visualization of vacuolar membranes (see also movies M4 to -6). Staining of germ tubes and hyphae resulted in almost immediate accumulation of the dye at the hyphal tip. This shows that *C. albicans* hyphae resemble in this respect hyphae of other filamentous ascomycetes. In a study by Crampin et al., a distinction based on molecular markers was drawn between polarisome components, for example, Cdc42, Spa2, and Bud6, and Spitzenkörper components, for example, Bni1 (8). In *A. nidulans*, the formin SEPA was shown to localize to the hyphal tip either as a crescent at the tip or as a spot near the tip, suggesting that SEPA is part of the Spitzenkörper (35). While in *S. cerevisiae* and in *A. nidulans* C-terminal tagging of formins appeared to result in functional fusion proteins, it appeared to be difficult to generate Bni1-GFP in *C. albicans* as the sole source of Bni1 (22). We as well as others found that Bni1-GFP colocalizes with the FM4-64-stained Spitzenkörper and in some cases simultaneously at the latest septal site as was also observed for SEPA in *A. nidulans*, suggesting mechanistic similarities (8, 22, 35). Very recently, Li et al. presented an analysis of *BNII* in *C. albicans*, which confirms our central findings of the role of Bni1.
in the regulation of cell polarity. Their report shows that Bni1 is required for correct alignment and positioning of the mitotic spindle and that \textit{bni1} defects correlate with the mislocalization of the \textit{C. albicans} Kar9 homolog (22).

In \textit{S. cerevisiae}, Bni1 was shown to form a polarisome complex with Spa2, Bud6, and Pea2 (36). In \textit{C. albicans}, Spa2 was localized in a manner similar to that of Bni1-GFP (44). This provides some further evidence for a conserved role of the polarisome in polarized hyphal growth. Interestingly, the \textit{C. albicans} genome encodes a \textit{BUD6} homolog (orf19.5087) but not a \textit{PEA2} homolog (at least according to assembly 19), which suggests that species-specific regulation of the central morphogenetic protein network exists in fungal species.

**Function of formins.** The function of formins in the assembly of linear actin filaments is conserved in nature and has elegantly been analyzed in several recent studies (12, 13, 33, 34).
Our analysis of the single formin deletion mutants did not reveal any drastic phenotypes in the assembly of the cortical actin cytoskeleton in growing yeast cells or germ tubes. Double deletion of both formin genes, \textit{BNI1} and \textit{BNR1}, in \textit{S. cerevisiae} results in synthetic lethality (22, 37), suggesting overlapping functions of both formins. This may also suggest a surrogate mechanism in which \textit{Bnr1} partially takes over \textit{Bni1} functions in \textit{C. albicans} (22). Since \textit{bnr1} strains are able to form mycelia, either \textit{Bnr1} does not contribute to polarized morphogenesis or its function can be taken over by \textit{Bni1}. We found that in a \textit{bnr1} deletion strain, cells are more elongated than in the wild type. Therefore, \textit{Bnr1} may have some function in the redirection of polarized secretion during septation which is promoted at a time when \textit{Bni1} may not localize to the bud neck and thus cannot take over \textit{Bnr1} function. \textit{Bni1}, however, is required for the maintenance of polarized hyphal growth. Both \textit{C. albicans} formins possess G protein binding and FH2 and FH3 domains corresponding to other Diaphanous-related formins. At the very C terminus, both \textit{C. albicans} formins also contain a small stretch that is conserved with other DADs, suggesting similar regulations of formins in \textit{C. albicans} by activation via G protein binding. The C-terminal localization of the DAD may explain the problems of generating C-terminal GFP fusions to \textit{BNI1} as the sole source of \textit{BNI1} in \textit{C. albicans}, which may require a longer linker region between the DAD and GFP [three copies of (Gly-Ala) repeats in our construct].

The \textit{bn1} mutant phenotypes resemble those of the \textit{SPA2} and \textit{WAL1} deletions. In \textit{bn1} and \textit{wal1} cells, polarized morphogenesis could be induced, but after germ tube formation, hyphal growth ceased and mycelial development was abolished. This is of biological significance, indicating that upstream events of signal perception and signal transduction are executed in these mutant strains but run into a morphogenetic block at the level of secretion and/or endocytosis (Fig. 8). As a response, polarized delivery of vesicles can be initiated, but subsequently defects in the organization of the actin cytoskeleton generate decreased polar growth rates and swelling of the germ tubes. The less drastic effects in \textit{bn1} cells than in \textit{spa2} and \textit{wal1} cells may in fact be the result of the \textit{BNR1} background and at least partial suppression (22). However, \textit{Bnr1} is almost 250 amino acids shorter than \textit{Bni1} and thus may lack...
some of the interaction potential of Bni1, which needs to be determined in future analyses.

Role of the actin cytoskeleton for polarized hyphal growth. Morphogenetic switching in C. albicans was shown to be dependent on Ras1 signaling and the activation of the transcriptional regulators Cph1 and Efg1 by a MAP kinase cascade and the cAMP pathway, respectively (4, 21, 23). In a previous study, we showed that defects in endocytosis due to the deletion of WAL1, which encodes the C. albicans homolog of the human WASP crippled mycelial development (40). In this study, we demonstrate a defect in the maintenance of hyphal growth due to the deletion of BN1. Bni1 is a direct effector protein of Rho protein signaling, whereas Wall lacks a G protein binding domain (in contrast to its human homolog) and is activated by an as-yet-unknown mechanism. Tip localization of both Bni1-GFP (this study and reference 22) and Spa2-GFP (44) suggests the presence of a polarisome complex in C. albicans hyphae. The role of Bni1 as part of the C. albicans polarisome is, therefore, the targeted delivery of secretory vesicles. Another conserved function of Bni1 is the formation of the actin ring at septal sites (potentially overlapping with Bnr1), and consistent with that Bni1-GFP was also found to localize to septal sites. The lack of a strong defect in mislocalization of cortical actin patches is consistent with a role of patches in endocytosis as a parallel pathway of the actin cytoskeleton. In our bni1 strains, cortical actin patches were polarized, although larger cells showed some mislocalization of patches to the mother cells, which was also observed by Li et al. (22).

In a recent study, an explanation of how transcription via Efg1 may influence growth decisions and activate actin cytoskeleton dynamics was provided. Under serum-inducing conditions, transcript levels of CDC24, encoding the guanine nucleotide exchange factor of Cdc42, transiently increase, which may result in an increase in Cdc42 activity that may trigger germ tube formation (3). Other genes involved in the organization of the actin cytoskeleton, e.g., RHO3 and BEM2, were found to be regulated in a similar manner (28).

Taken together, our results on the characterization of WASP and formins in C. albicans suggest a model of how regulatory networks signal to the actin cytoskeleton to promote growth decisions in C. albicans (Fig. 8). Our data also indicate that there are two largely separate pathways leading to secretion via formins and to endocytosis via WASP. Deletion of genes affecting either pathway will likely cripple the maintenance of polarized hyphal growth and may provide new means and targets for antifungal drug therapy to specifically perturb the hyphal growth phase in C. albicans.

Future research will be directed to elucidate how the interplay between secretion and endocytosis is regulated at the molecular level to achieve a balanced state of polarized cell growth.

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