Loss of Arp1, a putative actin-related protein, triggers filamentous and invasive growth and impairs pathogenicity in *Candida albicans*

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**A R T I C L E  I N F O**

**Article info**
Received 10 September 2020
Received in revised form 19 November 2020
Accepted 21 November 2020
Available online 01 December 2020

**Keywords:**
*Candida albicans*
Actin-related protein
Filamentous growth
Invasive growth
Pathogenicity
Nuclei separation
Spindle orientation
RNA sequencing

**A B S T R A C T**

The polymorphic cellular shape of *Candida albicans*, in particular the transition from a yeast to a filamentous form, is crucial for either commensals or life-threatening infections of the host. Various external or internal stimuli, including serum and nutrition starvation, have been shown to regulate filamentous growth primarily through two classical signaling pathways, the cAMP-PKA and the MAPK pathways. Genotoxic stress also induces filamentous growth, but through independent pathways, and little is known about negative regulation during this reversible morphological transition. In this study, we established that *ARP1* in *C. albicans*, similar to its homolog in *S. cerevisiae*, has a role in nuclei separation and spindle orientation. Deletion of *ARP1* generated filamentous and invasive growth as well as increased biofilm formation, accompanied by up-regulation of hyphae specific genes, such as *HWP1*, *UME6* and *ALS3*. The filamentous and invasive growth of the *ARP1* deletion strain was independent of transcription factors *Efg1*, *Cph1* and *Ume6*, but was suppressed by deleting checkpoint *BUB2* or overexpressing *NRG1*. Deletion of *ARP1* impaired the colonization of *Candida* cells in mice and also attenuated virulence in a mouse model. All the data suggest that loss of *ARP1* activates filamentous and invasive growth in vitro, and that it positively regulates virulence in vivo, which provides insight into actin-related morphology and pathogenicity in *C. albicans*.

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### 1. Introduction

*Candida albicans* is one of the most common fungal pathogens of humans, causing diseases ranging from superficial mucosal infections of immunocompetent individuals to life-threatening systemic infections in immunocompromised patients [1,2]. As a polymorphic fungus, the ability to switch from a yeast form to a filamentous form, containing either pseudohyphae, hyphae, or both, contributes to biofilm formation and to its pathogenicity [3,4]. Consistent with a role in pathogenicity, blocking the yeast-to-filamentous-form transition typically impairs both the ability of the pathogen to escape from immune cells, such as macrophages and neutrophils, as well as the ability to invade host tissues, resulting in attenuated virulence [5–7].

Numerous internal and external factors, including serum addition and nutrient starvation, induce filamentous growth of *C. albicans* cells. These signals act primarily through two main pathways, the cyclic-AMP/protein kinase A (cAMP-PKA) pathway and the mitogen-activated protein kinase (MAPK) pathway [6,8,9]. The cAMP/PKA pathway governs pathogenesis, morphological transition, as well as sexual reproduction and sporulation in several pathogenic fungi [10]. In *C. albicans*, the adenyl cyclase Cyr1 converts ATP to cAMP upon directly sensing environmental cues or being activated by Ras1; this cAMP can bind to the regulatory subunits of PKA (Bcy1) and liberate the catalytic subunits (Tpk1 and Tpk2) [8,11–12]. Downstream, the signal from the cAMP-PKA pathway works through Efg1, a key transcription factor, to initiate germ tube formation [13–14]. Deletion of *EFG1* results in a series of changed properties linked to pathogenicity, including decreased filamentous growth, reduced biofilm formation and attenuated cell virulence [15–17]. In contrast, the MAPK pathway signals through a cascade of kinases (Cst20, Ste11, Hst7, Cek1) and leads to the activation of transcription factor Cph1 [18,19].

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Genotoxic stresses can induce filamentous growth independent of the above two classic pathways in *C. albicans* [20]. Cells treated with genotoxic agents like hydroxyurea (HU) or methyl methanesulfonate (MMS) exhibit constitutive filamentous growth, while deletion of *RAD53*, the main checkpoint kinase, results in impaired filamentous growth during treatment with HU or MMS [20,21]. Furthermore, the inactivation of DNA damage repair usually generates abnormal cell morphology. For example, in *C. albicans* removal of a homologous recombination protein, Rad52 or a putative SUMO E3 ligase, Mms21, both lead to strong MMS sensitivity and also the induction of filamentous growth that is independent of transcription factor *EFG1* [22,23].

Currently, the investigation of the genes playing roles in inducing filamentous growth of *C. albicans* has achieved significant progress. By contrast, less is known about the negative elements which are also key to this morphology transition in *C. albicans*. In a previous study, we reported that several conditional mutants of *C. albicans* show invasive growth on non-hypal-inducing media through screening the GRACE (Gene Replacement And Conditional Expression) library, which is a collection of approximately 2500 conditional mutants created in the CaSS1 strain background with a purpose of finding essential genes and effective drug targets in *C. albicans* [23,24]. Among those invasiveness related genes, we identified a candidate actin-related gene, *ARP1*, which had not been assigned to any classic filamentous growth pathway and whose homolog in *S. cerevisiae* is required for spindle orientation and nuclear migration [25]. *ARP1* was also considered a potential DNA damage related gene based on our previous identification of MMS sensitive strains using the GRACE library [26].

In this study, we followed up on the role of *ARP1* in DNA damage and filamentous growth control, and characterized its function in morphological regulation in detail and explored its genetic interactions with classic filamentation regulating pathways in *C. albicans*. In general, we find that in *C. albicans* deleting *ARP1* triggers filamentous growth, invasive growth and biofilm formation, but leads to loss of pathogenicity in *vivo*.

2. Materials and methods

2.1. Strains, media, and reagents

*C. albicans* strains were grown in YPD medium with 50 mg uridine per liter as described [27]. MMS was purchased from Sigma (USA). HU and other chemicals were purchased from Sangon (China). Yeast nitrogen base (YNB) medium was supplemented with appropriate nutrients for identification of transformants by auxotrophic complementation [28]. Primers used in this study were listed in Table S1.

2.2. DNA manipulation

To construct an *ARP1* deletion strain in *C. albicans*, we used a transient CRISPR/Cas9 system as described [29]. Two homologous DNA fragments for *ARP1* were amplified with primers *ARP1*-Re-F1 and *ARP1*-Re-R1, as well as *ARP1*-Re-F2 and *ARP1*-Re-R2. The *HIS1* gene was amplified from plasmid pFHA103 with primers pF-A-F and pF-A-R. The repair DNA was then annealed by PCR using the two homologous DNA fragments and the *HIS1* gene as templates with primers *ARP1*-Re-F1 and *ARP1*-Re-R2. The Cas9 gene and sgRNA were amplified from plasmid pV1093 and then, together with repair DNA, transformed into the wild-type strain SN148 to replace the two alleles of *ARP1* with the *HIS1* marker as described [28]. The correct knockout strains were confirmed by PCR using primers *ARP1*-Te-F and *ARP1*-Te-R.

To rescue the phenotype of the *ARP1* deletion strain, a 2157-bp DNA fragment containing the full-length *ARP1* ORF as well as its promoter and terminator were amplified with primers *ARP1*-Te-F and *ARP1*-Te-R and cloned into the *KpnI* site of plasmid CIP10 [30], generating pCIP10-ARP1. Subsequently, pCIP10-ARP1 was linearized by Stul and integrated at the RP10 locus of the genome. The integration was confirmed by using primers URA3-TF and RPS-F.

To construct the *ARP1* *EFG1* double deletion strain, a previously constructed *EFG1* deletion strain [31] was used as the background strain to further delete the *ARP1* gene using the transient CRISPR/Cas9 system. To construct the *ARP1* *CPH1* and *ARP1* *UME6* double deletion strains, similar transient CRISPR/Cas9 systems for *CPH1* and *UME6* were used to delete *CPH1* or *UME6* in the *ARP1* deletion background.

To check the function of *NRG1* in the *ARP1*–related filamentous regulation, we replaced the original promoter of *NRG1* with a strong *ADH1* promoter to direct overexpression. An 803 bp *ADH1* promoter fragment was amplified with primers *ADH1*-F1 and *ADH1*-RH-B, while the 1302 bp ORF of *NRG1* was amplified with primers CaNRG1-RH-F and CaNRG1-R from the genome of *C. albicans*. The two DNA fragments representing the *ADH1* promoter and *NRG1* were annealed by PCR and then cloned onto XbaI and *PstI* sites in plasmid pCaEXP, generating pADH1–NRG1. Subsequently, pADH1–NRG1 was linearized by Stul and integrated at the RP10 locus. The integration was confirmed by PCR with primers RP10-F and ADH1-R-N. As well, *TUP1* was overexpressed in the *ARP1* deletion background using a similar protocol.

To increase the transcription of *ARP1*, we replaced the original promoter of *ARP1* with a *MET3* promoter, generating a heterozygous overexpression strain for *ARP1*.

To view the spindle morphology, a GFP tag was amplified from plasmid pFA-GFP-URA3 and then integrated at the 3′ end of *TUB2* gene.

2.3. Microscopy

To check the actin cytoskeleton, rhodamine-phalloidin staining was performed according to the manufacturer’s instructions (Beyotime, China). Generally, cells were fixed with 4% formaldehyde (v/v) and then washed three times with PBS buffer containing 0.1% Triton. Afterwards, cells were incubated in rhodamine-phalloidin dye (1/40 diluted with PBS buffer containing 0.1% Triton) overnight at 4°C. Finally, cells were washed twice with PBS buffer and imaged on a Leica DM5000B microscope (Leica Microsystems) with a 100 x objective. Spindle morphology assays were performed as previously described [32]. Generally, overnight cells were collected and fixed with 70% ethanol for 5 min, washed twice with 1 × PBS, incubated in 1.0 μg/ml DAPI for 10 min, and then washed twice with 1 × PBS before being mounted for analysis. Cells were imaged on a Leica DM5000B microscope (Leica Microsystems) with a 40 x objective.

2.4. Biofilm assay

Biofilm assays were performed as previously described [33]. Single colonies of the *ARP1* deletion strain and related strains were inoculated into 3 ml of YNB dextrose media and grown overnight at 30 °C. Each overnight culture was diluted to OD595 of 1.0 and 2 ml of diluted culture was added into a 24-well polystyrene plate (NEST), and kept for 48 h at 37 °C without shaking. The mature biofilm was washed and then stained by 0.4% w/v aqueous crystal violet. The destaining solution was 1/5 fold diluted in 95% ethanol (final volume of 200 μl) and the absorbance at OD595 was measured and averaged. The experiment was run in triplicate for each strain.
2.5. In vitro invasive assay

The overnight cultures were spotted on YPD plates with appropriate dilutions and kept at 30°C for 2–3 days. The colony morphology was imaged before washing with running water. The remaining cells on media after washing were considered as invasive growth cells.

2.6. RNA-seq assay

Single colonies of the C. albicans wild-type strain SN148 and the ARP1 deletion strain were each inoculated into 3 ml YPD and incubated overnight at 30 °C on a 220-rpm shaker. The overnight cultures were diluted to OD600 of 0.2 in 10 ml YPD media and grown to an OD600 of 0.8 at 30 °C with shaking, before harvested for RNA extraction.

The RNA extraction and sequencing were performed by Beijing Biomics Biotech Co.Ltd as before [28]. Generally, RNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA) and RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). Sequencing libraries were generated using NEB Next® Ultra® RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer’s protocols and index codes were added to attribute sequences to each sample. After cluster generation, the library preparations were sequenced on an Illumina Hiseq2500/X platform and 125/150 bp paired-end reads were generated. C. albicans SC5314, version A22 downloaded from the Candida Genome Database (CGD) (http://www.candidagenome.org/) was used as a reference genome. Raw data have been submitted to NCBI (Accession number: SAMN15795375, SAMN15795376, SAMN15795377, SAMN15815338, SAMN15815339, SAMN15815340).

2.7. In vivo invasive assay

To assess the invasive growth in vivo, colonization of the ARP1 deletion strain in organs was tested using a mouse model. Generally, six weeks old male ICR mice (n = 10) were inoculated via the tail vein with 200 µl of suspension with 1 × 10^6 C. albicans CFUs. After 48 h, the livers, spleens and kidneys of infected mice were taken out and ground. The homogenate was then spread on YPD plates for CFUs assessment. The number of total CFUs in each organ was normalized according to the weight of each organ.

2.8. Virulence studies

Male ICR mice of 6 weeks age (n = 10) were used for in vivo virulence studies as before [34]. Briefly, 200 µl of 6 × 10^6 ml^−1 cells were injected intravenously into the tail vein of 10 mice for each strain. Survival rates were checked daily and survival curves were generated according to the Kaplan–Meier method using the PRISM program (GraphPad Software). Sections were prepared from the kidneys of moribund mice and stained with periodic acid-Schiff’s (PAS) stain for histological examination [34]. Mouse studies were carried out under the guidelines established by the Ethics Committee of Nantong University, China (R20080556).

3. Results

3.1. Deletion of ARP1 causes defects in the actin cytoskeleton

We obtained the amino acid sequence of ARP1 (orf19.2641) from the genome database of C. albicans. Sequence alignment showed that CaArp1 has a high sequence identity (48.9%) with ScArp1 (Fig. S1-A). Protein domain analysis revealed that CaArp1 contains an actin domain (7–414aa), similar to ScArp1, suggesting the actin-related role of CaArp1 (Fig. S1-B).

We used rhodamine-phalloidin staining to check the effect of removal of the ARP1 gene on the actin cytoskeleton. In wild-type cells, actin patches were mainly observed as bright dots localizing to sites of polarized growth; in the ARP1 deletion cells, no clear actin patches, but rather a diffuse actin signal was observed (Fig. 1). Re-introduction of ARP1 gene into the ARP1 deletion strain reversed the defects in the actin cytoskeleton, suggesting an essential role of ARP1 in maintaining normal actin cytoskeleton.

3.2. Deletion of ARP1 generates defects in nuclei migration and spindle orientation

ARP genes in S. cerevisiae have diverse gene functions, among which ScArp1 shows a role in spindle orientation and migration of nuclei [25]. Given the strong functional and structural similarity of CaArp1 with ScArp1, we checked whether it is also playing a role in spindle function in C. albicans. In the present study, nuclei in large budded cells were stained with DAPI and monitored using a fluorescence microscope (Fig. 2-A). Most of the wild-type cells (86%) showed a normal nuclei migration, with 1 nucleus in the mother cell and 1 nucleus in the daughter cell, while the remaining wild-type cells had some level of non-standard nuclei positioning, with 12% of cells showing only 1 nucleus in the mother cell, and 2% of cells containing 2 separated nuclei in the mother cell without a nucleus in the daughter cells (Fig. 2-B). By contrast, 75% of ARP1 deletion cells with large buds showed abnormal nuclei positioning with 21% of cells containing 1 nucleus in the mother cell without a nucleus in the daughter cell, and 58% of cells containing 1 nucleus in the mother cell, with or without a nucleus in the daughter cell (Fig. 2-B). In general, only 21% of ARP1 deletion cells showed a classic distribution of mitotic nuclei. When a copy of ARP1 gene was introduced into the ARP1 deletion strain, the nuclei morphology was reversed to the pattern in the wild-type strain, showing 69% normal nuclei morphology.

Since improper migration of nuclei can reflect deregulated spindle function, we checked microtubules by tagging β-tubulin (Tub2) with GFP in both SN148 and in the ARP1 deletion strains. In wild-type, 21.1% of cells contained spindles, either in short bar-like spindles or long spindles that spanned mother and daughter cells, among which 3.6% of cells showed abnormal spindles, either confined in the mother cell or with wrong orientation (Fig. 2-C&D). In contrast, while a similar number (18.2%) of ARP1 deletion cells contained spindles, 16% of cells contained abnormal spindles, either confined in the mother cell or misoriented to the mother/bud axis. In addition, most of the remaining ARP1 deletion cells contained spindle pole bodies confined in the mother cell. As well, in the ARP1 complemented strain, 19% of cells showed normal spindles (Fig. 2-C&D), which is similar to the pattern in the wild-type strain. In general, our results suggest that Arp1 in C. albicans plays a critical role in nuclei migration and spindle orientation, similar to the function of ScArp1 in S. cerevisiae.

3.3. Deletion of ARP1 triggers invasive and filamentous growth of C. albicans

Through previous screening of the GRACE library, ARP1 was found to be connected with invasive growth and MMS-induced DNA damage response [23,26]. Cells deleted for ARP1 typically displayed a slow growth rate. Based on the growth curve (Fig. 3-A), we found that the ARP1 deletion strain showed an average doubling time of 2.7 h, while the wild-type strain showed an average doubling time of 1.7 h. As well, deletion of ARP1 generated an enlarged and elongated cell morphology in liquid media, where only 40.6% of the ARP1 deletion cells displayed typical yeast forms,
but 59.4% of the cells existed in elongated forms, either in pseudo-hyphae or polarized forms (Fig. 3-B). On solid media, the colonies of the ARP1 deletion strain showed rough surfaces and edges while the wild-type strain showed typical smooth surfaces and edges. When tested with a washing assay, the ARP1 deletion strain exhibited strong invasive growth into solid media while the wild-type strain rarely invaded into solid media (Fig. 3-C). The introduction of a single copy of the ARP1 gene into the ARP1 deletion strain was enough to reverse both its strong filamentous growth in liquid media and the enhanced invasive growth on solid media.

Since filamentous growth in C. albicans is associated with biofilm formation [35–36], which enhances virulence and increases resistance to antifungal drugs, we checked the ability of the ARP1 deletion strain to form biofilms. Consistently, the ARP1 deletion strains showed dramatically enhanced biofilm formation; close to 5.5 times that of the corresponding wild-type strain (Fig. 3-D).

3.4. ARP1 is involved in response to oxidative and cell wall stresses

We checked the potential function of Arp1 in response to various stresses. Similar to the actin-patch phenotype of an ARP2 deletion strain, deletion of ARP1 also resulted in a sensitive to cell-wall-stress phenotype, including sensitivity to 100 μg/mL CFW and 200 μg/mL Congo red (Fig. 4-A). As well, deletion of ARP1 rendered cells sensitive to 2.5 mM H2O2, to 10 ng/ml rapamycin as well as to 4 μg/mL fluconazole, which is consistent with the phenotype of ARP1 mutants in S. cerevisiae. Re-introduction of the wild-type ARP1 gene complemented those phenotypes of sensitivity to various stresses. However, deletion of ARP1 only caused a slight sensitivity to MMS, UV or HU (Fig. 4-B), suggesting a minor role of ARP1 in the response to genotoxic stress.

3.5. Transcription profiling of ARP1 deletion

To better understand the function of the ARP1 gene, we assayed the pattern of gene transcription upon deleting ARP1. Overall, we found 220 genes (FDR < 0.05, Log2 fold > 1.5 or < -1.5) whose levels of transcription were significantly affected by loss of ARP1, with 203 genes up-regulated and only 17 genes down-regulated, suggesting a generally negative role of ARP1 on global gene expression (Table S2, Fig. S2). GO term analysis identified that the majority of affected genes were involved in ribosome and ribonucleoprotein...
complex biogenesis, rRNA metabolic processes, and carbohydrate transport. Moreover, we found 25 out of 220 genes showing altered transcription are related to oxidoreductase functions (Fig. 5-A). A majority of these genes, including \textit{HPD1}, \textit{FDH1}, orf19.1438, IDP2, orf19.2394, ADH2, ALD6, orf19.1117, ALD4, AYR3, orf19.1639, encode dehydrogenases. The remaining genes, including \textit{CFL2}, \textit{FRE7}, CFL5, CFL4, FRE7, RNK3, ARG5,6, mainly encode reductases. The modified transcription of these oxidoreductase-related genes may damage the redox homeostasis inside cells, supplying a potential explanation for the dramatic sensitivity to H$_2$O$_2$ caused by deleting \textit{ARP1}.

We also found that a number of genes that were significantly changed were correlated with filamentous and invasive growth (Fig. 5-B). \textit{HWP1}, encoding a classic hyphal cell wall protein [37], shows the highest transcriptional induction (5.7 fold), which is consistent with the observed increase in filamentous growth. As well, transcription factor \textit{UME6} (3.6 fold) and \textit{SFL2} (3.6 fold) are also up-regulated, but no other cAMP-PKA or MAPK pathway-related transcription factors, like \textit{EFG1} or \textit{CPH1}, are significantly changed in expression [38–39]. In addition, the up-regulation of \textit{HGC1} (2.0 fold), which encodes a hypha-specific G1 cyclin-related protein involved in the regulation of morphogenesis [38,40], may identify a potential cell cycle related role of \textit{ARP1} in filamentous regulation.

Consistent with the increased biofilm formation of the \textit{ARP1} deletion strain, we also found the up-regulation of a series of specific genes, including \textit{ALS5} (3.5 fold), \textit{ALS1} (2.3 fold) [41–42] and biofilm-related genes, \textit{MRV8} (3.7 fold), and \textit{RBT1} (3.3 fold) [43–44]. Similarly, the up-regulation of \textit{JEN2} (4.6 fold), and \textit{NAG4} (2.8 fold) may suggest a possible underlying mechanism of the strong invasive growth of the \textit{ARP1} deletion strain [45].

3.6. Interactions between \textit{ARP1} deletion and filament-controlling transcription factors

Based on the RNAseq data, deletion of \textit{ARP1} did not influence the transcription of either \textit{EFG1} or \textit{CPH1}, the two main transcription factors downstream of the cAMP-PKA and MAPK pathways, but up-regulated the transcription of \textit{UME6}, a transcription factor involved in hyphal elongation. To test whether the filamentous growth caused by deleting \textit{ARP1} is related to the cAMP-PKA or the MAPK pathways, we deleted either \textit{EFG1}, \textit{UME6} or \textit{CPH1} in the \textit{ARP1} deletion background. As shown in Fig. 6-A, in liquid YPD media, the \textit{ARP1} deletion strain mainly showed filamentous forms, while the \textit{EFG1} deletion strain, the \textit{UME6} deletion strain, and the \textit{CPH1} deletion strain primarily existed in yeast forms. Disrupting \textit{EFG1} or \textit{CPH1} in the \textit{ARP1} deletion background did not block the filamentous growth characteristic of the \textit{ARP1} mutant strain, and in fact somewhat enhanced the frequency of elongated cells, a surprising consequence for the removal of hyphal-directing transcription factors. Furthermore, deleting \textit{UME6} in the \textit{ARP1} disrupted background only slightly decreased filamentous growth (44.5% vs 54.6%, P = 0.112), suggesting that \textit{UME6} may also not be essential for the filamentous growth triggered by deleting \textit{ARP1}. 

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**Fig. 2.** Deleting \textit{ARP1} generates defects in nuclei migration and spindle orientation. (A) The nuclei morphology in wild-type strain (SN148), the \textit{ARP1} deletion strain and the \textit{ARP1} complemented strain were checked through DAPI staining. (B) The large budded cells with nuclei structures that fell into one of the three categories (one nucleus in mother cell and one nucleus in daughter cell, only one nucleus in mother cell, and two or more nuclei in mother cell either with or without one nucleus in daughter cell) were identified, and the relative fraction of each category was determined. N \geq 100. (C) Wild-type strain (SN148), the \textit{ARP1} deletion strain and the \textit{ARP1} complemented strain carrying a Tub2-GFP tag, were grown in liquid YPD overnight to check the spindle morphology. In wild-type strain and the \textit{ARP1} complemented strain, the spindle typically spanned the mother cell and daughter cell, while in the \textit{ARP1} deletion strain the spindles were confined in mother cells with an angle to the mother/bud axis. (D) The large budded cells with indicated spindle structures were counted. N \geq 137.
Fig. 3. Deletion of ARPI causes increased filamentous growth and invasive growth as well as biofilm formation in *C. albicans*. (A) Growth curves of wild-type strain (SN148), the ARPI deletion strain and the ARPI complemented strain were made by measuring the OD595 at the indicated time points. For each strain, 3 different colonies were tested and the average data was shown. (B) Both wild-type strain, the ARPI deletion strain and the ARPI complemented strain were grown in liquid YPD and imaged on a microscope (Uppanel). Cells in yeast form or filamentous form were determined by microscopy of cells grown in YPD (Down panel). (C) Wild-type strain, the ARPI deletion strain and the ARPI complemented strain were spotted on solid YPD plates, and the colony morphology before or after washing was imaged using a camera. (D) Biofilm formation assay of wild-type strain, the ARPI deletion strain and the ARPI complemented strain. The indicated cells were grown to the stationary phase in liquid YNB media at 30 °C, diluted to OD595 = 1 and transferred to a 24 well plate and kept at 37 °C for 48 h to form biofilm. Cells were washed with PBS and stained with crystal violet. The absorbance of de-staining solution at OD595 was measured and averaged. Error bars = standard deviation. *p < 0.05, two-tailed t test.

Fig. 4. Phenotypic assay of the ARPI deletion in *C. albicans*. Deletion of ARPI resulted in sensitivity to oxidative stress, cell wall stress as well as rapamycin and fluconazole (A), but slight sensitivity to genotoxic stress (B). Wild-type strain (WT + CIP10), the ARPI deletion strain (ARPIΔ/Δ+CIP10), and the complemented strain (ARPIΔ/Δ+CIP10-ARPI) were used for phenotypic assay.
On solid media, the ARP1 deleted cells formed colonies with rough surfaces and edges while the EFG1, UME6 and CPH1 deletion strains showed standard smooth and regular surfaces and edges (Fig. 6-B). All the double mutants showed irregular surfaces and edges, similar to the ARP1 deletion cells. Consistently, double deletions of ARP1 with UME6 or CPH1 showed invasive growth into solid media typical of the ARP1 deletion strain, while the ARP1 EFG1 double deletion strain showed somewhat reduced invasive ness (Fig. 6-B). The present data may suggest the invasive growth is not directly related to the filamentous growth triggered by deleting ARP1.

3.7 The filamentous and invasive growth triggered by deleting ARP1 is suppressed by deleting checkpoint BUB2

Recent studies reported that DNA checkpoint proteins, such as Rad53, are involved in inducing filamentous growth, so we asked whether filamentous growth triggered by deleting ARP1 depends on checkpoints. We assessed genome stability, found that ARP1 deleted cells carrying a heterozygous URA3 gene at the RP10 locus increased the number of URA3 auxotrophs on 5-FOA plates to nearly 30 times the level seen for the wild-type strain, suggesting a severe reduction in mitotic genome stability was caused by deleting ARP1 (Fig. S3).

This severe genome instability suggests that the filamentous growth caused by deleting ARP1 could be checkpoint related. Therefore, we deleted RAD53 in the ARP1 deletion background to check the cell morphology. The RAD53 deletion strain showed normal yeast form growth with an increased number of attached cells, implicating a cell-cycle dysfunction, but deleting RAD53 in the ARP1 deletion background showed a similar filamentous morphology to the cells deleted with ARP1 alone (Fig. 7-A). On solid media, the RAD53 deletion strain formed smooth and regular colonies. In contrast, the RAD53 ARP1 double deletion strain showed rough and irregular surfaces and edges, as well as invasive growth into solid agar media, similar to the ARP1 deleted strain, also suggesting an independent role of Arp1 from Rad53.

Given Arp1 has a role in maintaining normal spindle orientation both in S. cerevisiae and C. albicans, we investigated whether Arp1 could show an involvement with the spindle checkpoint factor Bub2. We found that deletion of BUB2 in the ARP1 deletion strain significantly blocked its filamentous growth; only around 12.6% of the double deletion strain for ARP1 and BUB2 showed filamentous forms, which is significantly lower than the level in the ARP1 mutant alone (P < 0.01). In addition, the invasive growth of
the ARP1 deletion strain on solid YPD was significantly alleviated by further deleting BUB2 (Fig. 7-B). Taken together, the current data suggests that ARP1-related filamentous growth relies significantly on the checkpoint protein Bub2.

3.8. Nrg1 plays redundant roles with Arp1 in filamentous growth and biofilm formation

Since loss of Arp1 triggers filamentous growth, we investigated its relationship with identified negative regulators of filamentous growth. NRG1 is a general negative regulator of filamentous growth; therefore, we asked whether increasing the expression of NRG1 can suppress the filamentous growth induced by deleting ARP1. Here, we used an ADH1 promoter to up-regulate the expression of NRG1. In non-hyphae-inducing media, overexpressing NRG1 reduced the filamentous growth of the ARP1 deletion strain, resulting in fewer elongated and connected cells. On solid media, overexpressing NRG1 in the ARP1 deleted background reversed the rough colony morphology caused by deleting ARP1, to a smooth one similar to that of the wild-type strain (Fig. 8-A). After washing, only the ARP1 deletion strain showed enhanced invasive growth, while the ARP1 deletion strain carrying ADH1p-NRG1 showed reduced invasive growth, similar to that of the wild-type strain (Fig. 8-A).

In biofilm assays, overexpressing NRG1 in the ARP1 deleted background significantly reduced biofilm formation, resulting in levels even lower than that of the wild-type strain (Fig. 8-B). Therefore, the results suggest an inhibitory function of NRG1 on biofilm formation caused by deleting ARP1.

3.9. ARP1 deletion causes decreased colonization in vivo

Since deleting ARP1 induced filamentous growth and increased invasive growth in vitro, we wonder whether its deletion may increase invasiveness in vivo and result in enhanced colonization in tissues and organs. To check this possibility, identical amounts of wild-type and the ARP1 deletion cells, as well as the ARP1 complement cells, were injected into mice (n = 10 for each strain), and the CFUs in different organs were checked (Fig. 9-A&B&C). Unexpectedly, no increased invasiveness, but a global decreased colonization of the ARP1 deletion strain in organs was found. In livers, around 10^8 wild-type CFUs were found but only 194 CFUs for the ARP1 deletion strain were found; the introduction of a single copy of ARP1 gene into the ARP1 deletion strain rescued the defects in colonization. Similar colonization defects caused by deleting the ARP1 gene were found in spleens and also kidneys, suggesting a comprehensive impairment in invasive growth in vivo.

3.10. ARP1 deletion attenuates virulence in a mouse model

The heightened invasiveness in vitro, but the defect in colonization in vivo compelled us to test the virulence of the ARP1 deleted strain. Wild-type cells and ARP1 deletion cells were separately injected into mice, and the survival of the infected mice was monitored. The mice infected with wild-type cells all died by day 9, showing an average survival of 6 days, while the mice infected with ARP1 deletion cells were all alive on day 15, establishing the
Fig. 7. Filamentous and invasive growth of the ARP1 deletion are dependent on checkpoint Bub2. The indicated strains were grown overnight in liquid YPD at 30 °C before imaged using microscopy (Left panel). The percentage of filamentous cells was listed at the left of the image (3 independent colonies for each strain, n> 200). The liquid culture of represented strains was spotted on YPD plates and kept at 30 °C for 2–3 days. The invasive growth was checked by washing (Right panel).
Fig. 8. Overexpressing NRG1 reversed the filamentous and invasive growth of the ARP1 deletion. (A) Cell morphology and colony morphology of the ARP1 deletion strain with or without overexpressing of NRG1, in liquid YPD or on Solid YPD media. (B) Biofilm formation of the ARP1 deletion strain with or without overexpressing NRG1. The experiments were assayed in triplicate. Two independent ARP1 strains carrying ADH1p-NRG1 were used and compared with the ARP1 deletion strain. Two tail paired t-test, *P < 0.05.

Fig. 9. Deleting ARP1 reduces colonization and virulence in mice. (A-C) The indicated strains with same amount (1 × 10⁶) of CFUs were injected into a single mouse (n = 10) and kept for 48 h. The livers, spleens and kidneys were ground and spread on YPD plates for CFU assays. The number of CFUs in each organ was normalized to the weight of indicated organs (CFUs per gram). Paired t-test was used to compare the difference between different groups. (D) Survival curves of mice intravenously infected with indicated C. albicans strains. Male ICR mice (6 weeks old, 10 mice for each group) were injected with 1.2 × 10⁶ stationary-phase cells. Mice were checked daily for morbidity. (E) Histopathological examination of kidney tissues of moribund mice was obtained from moribund mice after infection. The infected kidney tissues were stained with periodic acid-Schiff’s reagent. The hyphae were indicated by arrows.
avirulence of the ARP1 deletion cells (Fig. 9-D). Consistently, in the kidneys of mice infected with wild-type cells, a mass of filamentous cells was found, but not in the mice infected with ARPI deletion cells (Fig. 9-E).

4. Discussion

Filamentous growth of C. albicans is critical for the fungus to be a successful pathogen, in part because the process appears important for invasion into host tissues, thereby causing life-threatening infections. This morphological transition is reversible, and is regulated by both positive and negative regulators. Currently, numerous hyphae specific regulators, including HWP1, EFG1 and UME6, have been identified as inducing or maintaining filamentous growth. Moreover, several negative regulators, including NRG1 and TUP1, have also been identified to inhibit filamentous growth by suppressing the expression of hypha-specific genes [46–47]. In this study, we identified that inactivation but not overexpression of ARPI (Fig. S4), a putative actin-related protein, triggers filamentous growth and invasion of agar surfaces, but blocks pathogenicity in a mouse model of candidiasis.

Actin-related proteins represent a protein family in eukaryotic cells that is involved in such diverse processes as cell motility, cytokinesis, vesicle transport, and chromatin remodelling [48–50]. In S. cerevisiae, the ARP family represents a set of actin-related-protein encoding genes, ranging from ARPI to ARP10. These actin-related proteins have been found to play diverse functions in various model organisms: in S. cerevisiae, Arp1 is required for nuclei migration and cell wall integrity checkpoint functions [25], in Aspergillus fumigatus, Arp1 modulates conidial pigmentation and complement deposition [51–52], in Plasmodium berghei, Arp1 appears essential and critical for vesicular transport [48]. In S. cerevisiae and C. albicans, the Arp2/Arp3 complex is a highly conserved actin nucleation center required for the motility and integrity of actin patches and is involved in endocytosis and membrane growth [53–54]. In this study, we found the removal of Arp1 in C. albicans impairs normal actin cytoskeleton, nuclei migration and spindle orientation as well as increases sensitivity to cell wall stresses, suggesting conserved roles of Arp1 and its orthologs in these model organisms.

The link between the essential role of Arp1 in spindle orientation and the abnormal filamentous morphology was somewhat unexpected. The defects in nuclei segregation and spindle orientation suggest a cell-cycle related function of Arp1. Previously, numerous studies revealed that blocking the cell cycle progression induces filamentous growth [55–56]. Cells either treated with HU or MMS, or deleted with the protein phosphatase 4 that is involved in deactivation of checkpoint kinase Rad53, promote filamentous growth [34,57–58]. As well, depletion of Hsp90 leads to destabilized cyclin-dependent kinase Cdcd28 and filamentous growth, suggesting a potential link between Hsp90, morphogenesis, and cell cycle progression [59]. Furthermore, the inactivation of DNA damage repair progress usually generates abnormal cell morphology. For example, removal of a homologous recombination protein, Rad52, leads to both strong MMS sensitivity and the induction of filamentous growth [22]. Recently, we reported Mms21, a putative SUMO E3 ligase with a role in maintaining genome integrity, negatively regulates invasiveness and filamentation independent of transcription factor EFG1 in C. albicans [23]. And, similar to the ARPI deletion, depletion of the dynein heavy-chain gene DYN1 leads to aberrant nuclear positioning and filamentous growth [60], whereas further deleting BUB2, a spindle position checkpoint, suppresses the pseudohyphal-like growth of DYN1 deletion, suggesting the spindle checkpoint may play critical roles in regulating filamentous growth [61]. In our study, the increased genome instability and disordered spindle orientation imply a potential link between Arp1 and checkpoint proteins such as Rad53 or Bub2. And in fact, in the ARPI deletion background further deletion of BUB2, but not RAD53, significantly suppressed its filamentous and invasive growth, suggesting a specific linkage of Arp1 to spindle checkpoint factor Bub2. Therefore, it is possible that deletion of ARPI impairs normal nuclei and spindle structure, and activates a Bub2 related checkpoint to induce cell polarization, resulting in filamentous growth.

We also observed that deletion of the ARP2/ARP3 complex in C. albicans generates round and swollen yeast phase cells in hyphae-inducing media and causes a global lack of induction of hyphae-specific genes upon the yeast-to-hyphae switch [62]. As well, Arp4 plays a role in hyphae formation since it is a subunit of the Nul4 histone acetyltransferase complex and is recruited by Efg1 to the promoters of hypha-specific genes [63]. It will also be interesting to test the contradictory roles between ARP1 and Arp2/Arp3 complex in regulating filamentation.

How does deleting ARPI activate filamentous growth? First, we performed transcription sequencing, which showed a global up-regulation of hyphae-specific genes, including HWP1, RB5 and ALS3. Although UME6, a transcription factor in maintaining true hyphal elongation [38,64], was up-regulated, EFG1 and CPH1, the main downstream elements of the cAMP-PKA and the MAPK pathways, were not found to be up-regulated, suggesting the strong filamentous growth triggered by deleting ARPI was not directed by either the cAMP-PKA or the MAPK pathways. Consistent with this prediction, deleting either UME6, CPH1 or EFG1 in the ARPI deletion background did not block filamentation, supporting the filamentous growth triggered by deleting ARPI is not activated by the MAPK pathway or the cAMP/PKA pathway. Strikingly, deletion of either EFG1 or CPH1 in the ARPI deletion background slightly increased the filamentous growth, which may suggest the negative roles of Cph1 and Efg1 in this Arp1-inactivation-mediated filamentation. The unexpected negative role of Efg1 and Cph1 seems contradictory to their function in regulating hyphae-specific gene expression during filamentous growth. Perhaps dysregulation of gene expression resulting from loss of the transcription factors enhances a filamentation signal resulting from the loss of Arp1; transcription profiling of the double mutants could determine if any hyphal-inducing signals are enhanced. And indeed, we found some clues to support the negative role of EFG1 in filamentous growth; deletion of ARPI increases filamentation during the growth within the matrix, which may supply similar underlying mechanism [65]. Taken together, Ume6, Efg1 and Cph1, the main downstream transcription factors of cAMP-PKA and MAPK signalling pathways, are dispensable in ARP1 related filamentous growth.

A possible clue is the filamentous growth inhibitor, NRG1. Over-expressing NRG1, but not its coactivator TUP1 (Fig. S5), is effective in partially blocking the filamentous growth induced by deleting ARPI. Since NRG1 represses filamentous growth mainly via suppressing the expression of hypha-specific genes, including HYRI, ALS8, HWP1 and ECE1, we could expect they function on common targets, such as HWP1. In addition, the up-regulation of HGC1, a hypha-specific G1 cyclin-related protein may also suggest the filamentous growth triggered by deleting ARPI is checkpoint-related. Taken together, a possible signaling pathway for Arp1 in activating filamentous growth could be that Arp1 functions in maintaining normal nuclei segregation and spindle orientation, whereas its deletion may block normal cell cycle progress through a Bub2 related checkpoint and cause a direct or indirect effect in up-regulating the expression of certain hypha-specific genes such as HWP1, resulting in filamentous growth.

Strong filamentous growth and invasive growth of C. albicans usually indicate increased invasiveness in hosts, resulting in
enhanced tissue damage and pathogenicity. Intriguingly, in our study, although strong invasive growth upon deleting ARP1 was found in vitro, no similar invasive growth was found in vivo. The contradictory results suggest a comprehensive regulation of virulence that may contain various external and internal factors. Primarily, the disordered cell morphology could be one of the reasons. Upon deleting ARP1, around 60% of cells are blocked in filamentous form; therefore, their distribution in hosts and the virulence may be impaired. Consistent with this, a similar filamentous morphology and decreased virulence were found by deleting RAD52 [66]. As well, although 40% of the ARP1 deletion cells are in yeast form, they have defects in nuclei separation and spindle orientation, suggesting the poor status which may also contribute to abnormal cell morphology from generation to next generation.

The strong sensitivity to oxidative stress by deleting ARP1 could also be an important factor for impaired virulence. To invade host tissues, C. albicans cells need to break through the first line of immune system. Otherwise, the filamentous growth or invasive ability is insignificant for the virulence, like for the ARP1 deletion. In view of this, the ARP1 deletion strain may be more susceptible to killing by ROS-producing immune cells such as macrophages and neutrophils. To support this idea, we tested the internal ROS and SOD activity and found that the SOD activity in ARP1 deletion cells is nearly 3 times to the level in wild-type strain (data not shown), suggesting disordered redox homeostasis. Similar results have been noted; for example, deleting RTT109 not only causes strong filamentous growth as well as increased sensitivity to H2O2, but also results in significantly less pathogenicity in mice and a greater susceptibility to killing by macrophages that can be suppressed by adding an oxidative inhibitor [67]. The similarity between Arp1 and Rtt109 is that they both function in maintaining genome stability that may suggest potential cell cycle-related morphology regulating mechanisms. Similarly, in our previous study, PTC2 PPH3 double deletion promotes filamentous growth in hyphae-inducing media, but results in decreased cell virulence, compared with their single deletions, probably due to the strong sensitivity to oxidative and genotoxic stresses [68]. Therefore, we can suggest that increased filamentous growth in vitro, in some cases, is not associated with strong invasiveness and pathogenicity in vivo.

In general, we find that loss of ARP1 activates filamentous and invasive growth, which is independent of known cAMP/PKA or MAPK signaling pathways, but is dependent on spindle checkpoint Bub2 (Fig. 10). A general hyphae inhibitor, NRG1, plays a redundant role in ARP1-induced filamentation, invasiveness and biofilm formation. In addition, the increased invasiveness upon ARP1 deletion does not result in increased pathogenicity, probably because of the poor survivability in the host. Our research links the putative actin-related protein Arp1, to morphological regulation and pathogenicity. But the signaling pathway from ARP1 to filamentous growth and pathogenicity remains unclear. Further studies are needed to explore the downstream mechanism of ARP1 related morphology regulation and also the possibility of ARP1 for drug therapy.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the National Natural Science Foundation of China to J.F. (No. 82072267), NSERC grants to M.W. (CRC 950-228957 and discovery RGPIN/4799), the Priority Academic Program Development of Jiangsu Higher Education Institutions and the Jiangsu government scholarship for overseas studies, China. We thank Merck for making the Grace library available to the scientific community.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.csbj.2020.11.034.

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