Mycobiology

ISSN: 1229-8093 (Print) 2092-9323 (Online) Journal homepage: https://www.tandfonline.com/loi/tmyb20

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To cite this article: Jeongmi Kim, Minji Park, Eunsoo Do & Won Hee Jung (2014) Mitochondrial Protein Nfu1 Influences Homeostasis of Essential Metals in the Human Fungal Pathogen *Cryptococcus neoformans*, Mycobiology, 42:4, 427-431, DOI: 10.5941/MYCO.2014.42.4.427

To link to this article: https://doi.org/10.5941/MYCO.2014.42.4.427

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Published online: 19 Jun 2018.

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Mycobiology

Research Note

Mitochondrial Protein Nfu1 Influences Homeostasis of Essential Metals in the Human Fungal Pathogen *Cryptococcus neoformans*

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Abstract Mitochondrial protein Nfu1 plays an important role in the assembly of mitochondrial Fe-S clusters and intracellular iron homeostasis in the model yeast *Saccharomyces cerevisiae*. In this study, we identified the Nfu1 ortholog in the human fungal pathogen *Cryptococcus neoformans*. Our data showed that *C. neoformans* Nfu1 localized in the mitochondria and influenced homeostasis of essential metals such as iron, copper and manganese. Marked growth defects were observed in the mutant lacking *NFU1*, which suggests a critical role of Nfu1 in Fe-S cluster biosynthesis and intracellular metal homeostasis in *C. neoformans*.

Keywords *Cryptococcus neoformans*, Fe-S cluster, Metals, Mitochondria, Nfu1

Fe-S clusters are ubiquitous cofactors present in most organisms. Proteins containing the Fe-S cluster play critical roles in various cellular processes, including respiration and regulation of gene expression. Fe-S cluster biosynthesis is highly conserved in prokaryotes and eukaryotes. Three systems are involved in the assembly of Fe-S clusters in prokaryotes. Nitrogen fixation is responsible for the assembly of the Fe-S cluster for nitrogenase in nitrogen-fixing bacteria, and the Fe-S clusters and sulfur-mobilization assembly machineries synthesize Fe-S clusters required for proteins with house-keeping functions and stress response [1-3]. In eukaryotes, mitochondrial Fe-S cluster assembly, Fe-S cluster export and cytosolic Fe-S cluster assembly systems are three key machineries for Fe-S cluster biosynthesis. Mitochondrial functions of the Fe-S cluster assembly are particularly important for cellular Fe-S cluster biosynthesis, whereas Fe-S cluster export and cytosolic Fe-S cluster assembly systems are only involved in cytosolic Fe-S cluster biosynthesis. In the model yeast *Saccharomyces cerevisiae*, 14 proteins have been identified to be required for the mitochondrial Fe-S cluster assembly system [4].

Fe-S cluster biosynthesis is not only critical for enzymes required for house-keeping functions but is also involved in virulence in a number of bacterial pathogens, including *Shigella flexneri* and *Erwinia chrysanthemi* [5, 6]. Any direct contribution of Fe-S cluster biosynthesis in the virulence of fungal pathogens has not been demonstrated. However, recent studies have shown that Fe-S cluster biosynthesis is mainly regulated by the conserved Hap protein complex, and that deletion of the Hap protein homolog influences the virulence of fungal pathogens *Candida albicans*, *Aspergillus fumigatus*, and *Cryptococcus neoformans* [7-9].

The basidiomycete fungus *C. neoformans* is the causative agent of life-threatening cryptococcosis and mostly infects patients who are immunocompromised owing to human immunodeficiency virus infection, cancers or organ transplantation [10, 11]. Like most other microbial pathogens, iron acquisition and regulation play essential roles in survival and the expression of virulence factors of *C. neoformans* within the host [12, 13]. Transcription factors that are responsible for the regulation of iron acquisition have been identified, and their involvement in controlling the expression of genes required for Fe-S cluster biosynthesis have been suggested [9, 14]. The protein encoded by the gene CNAG_03395 in the genome of *C. neoformans* var. *grubii* H99 strain (serotype A) is an example. This protein has a putative function in Fe-S cluster biosynthesis, and its transcript levels were found to be significantly down-regulated in a mutant lacking the gene encoding the iron regulatory protein HapX [9]. The protein encoded by CNAG_03395 is

Mycobiology 2014 December, **42**(4): 427-431
http://dx.doi.org/10.5941/MYCO.2014.42.4.427
pISSN 1229-8093 • eISSN 2092-9323
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Received October 28, 2014
Revised November 13, 2014
Accepted November 20, 2014

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highly homologous to NifU of nitrogen-fixing bacteria. Its C-terminal domain (amino acids 189-249) shows 38% identity to *Azotobacter vinelandii* NifU, which functions in Fe-S cluster assembly for the maturation of nitrogenase components [15-17]. *S. cerevisiae* also possesses the NifU homolog Nfu1, which also shows 38% identity to *A. vinelandii* NifU. Nfu1 displays synthetic lethality with Ssq1, which is a mitochondrial heat shock protein 70 and is involved in Fe-S cluster assembly [18]. In addition to Nfu1, two other bacterial NifU-like proteins, Isu1 and Isu2, have been found in *S. cerevisiae*. Furthermore, it has been suggested that Nfu1 localizes in mitochondria, and together with Isu1, plays roles in mitochondrial Fe-S cluster assembly and iron homeostasis in *S. cerevisiae* [16].

Our previous findings and the importance of Nfu1 in *S. cerevisiae* led us to characterize a function of the Nfu1 ortholog in *C. neoformans*. Because *S. cerevisiae* Nfu1 plays a role in mitochondria, we first investigated the localization of the protein. The C-terminal of Nfu1 was fused with the 3×FLAG epitope tag (DYKDDDDK) and introduced into the *C. neoformans* var. *grubii* H99 strain by homologous recombination. Cryptococcal cells expressing the Nfu1-FLAG fusion protein were grown in yeast extract peptone destrose medium, followed by isolation of the mitochondrial fraction as described elsewhere, and western blot analysis was performed using the anti-FLAG antibody [19]. As shown in Fig. 1A, the western blot data using the isolated mitochondrial fraction suggested that Nfu1 localizes in mitochondria in *C. neoformans*. Moreover, our data revealed that Nfu1-FLAG protein levels are also influenced by iron levels in the medium (Fig. 1B).

For functional characterization of Nfu1 in *C. neoformans* var. *grubii* H99, a gene-specific deletion cassette was prepared by overlap PCR using primers Nfu1-KO1 (CTCCAGCAC-AATATATGCCCTGGTTAC), Nfu1-KO2 (AATTCTGCA-GATATCCATCACACTGGCGGCCGAAATGGGCGGAC-ATATGGCAATATC), Nfu1-KO3 (AATTCCAGCACACT-GGCGGCCGTTACTAGTGGGTTCGCTGGTTTTGCGA-CCTAATAC), Nfu1-KO4 (GCGACGCCAAACCCATCTT-TCACAATATG), Nfu1-KO5 (TCAAGATGTGACCAGCA-CCGATTGAC) and Nfu1-KO6 (GGAGCCCCAATATCT-CCCTTTTTGAC) with genomic DNA and the plasmid pJAF1 containing the neomycin resistance marker as templates [20, 21]. The wild-type strain was biolistically transformed with the amplified gene deletion cassette as described previously [22]. Three independent positive transformants were selected, confirmed by Southern blot analysis and used throughout this study (Fig. 1C). For construction of the strain with reintroduced *NFU1*, the wild-type *NFU1* gene was amplified by PCR using primers Nfu1-Re1 (GGCGACCTCCGAACATTGTAATTGTCATC) and Nfu1-Re2 (GAACAGGAACTCTCGAAGATGGATCAG), cloned into the plasmid pCH233 containing the nourseothricin resistance marker, and transformed into two independent *nfu1* mutants. Positive transformants containing wild-type *NFU1* at its original locus were identified by PCR and

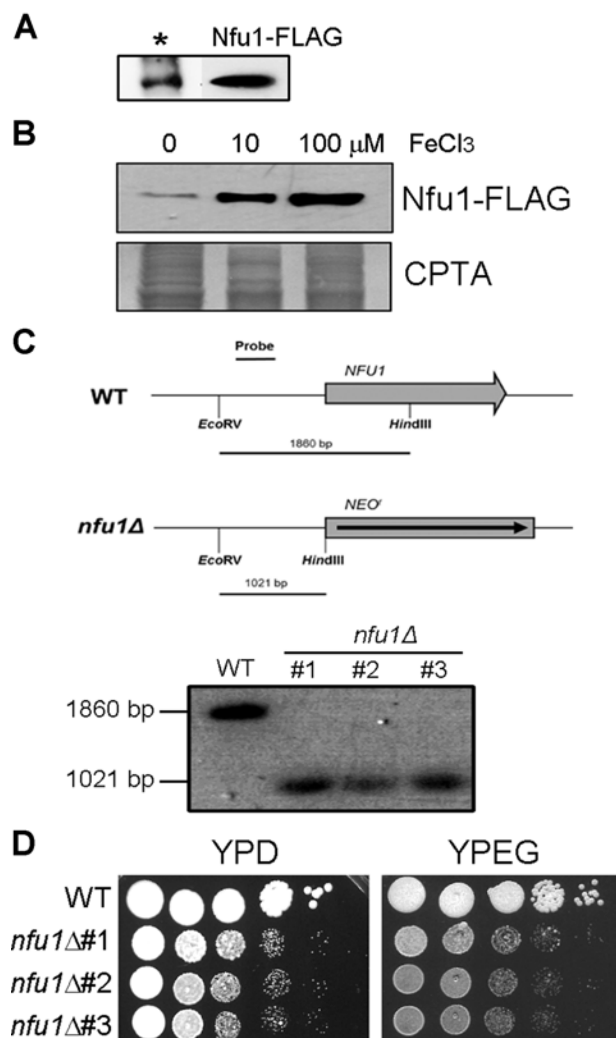

Fig. 1. Nfu1 localized in mitochondria, and the mutant lacking *NFU1* showed reduced growth. A, Western blot analysis was performed with isolated mitochondrial fractions using the anti-FLAG antibody. The asterisk indicates the mitochondrial Lys4-FLAG fusion protein, which was used as a reference; B, The abundance of the Nfu1-FLAG protein in the cells grown in medium containing different concentrations of $FeCl_3$ (0, 10, and 100 mM) was evaluated by western blot analysis using the anti-FLAG antibody. The same protein samples were stained with copper phthalocyanine-3,4′,4″,4‴-tetrasulfonic acid tetrasodium (CPTA) to show equal loading of each sample. The results of Southern blot analysis; C, Genomic DNA of the wild type and *nfu1* mutants were digested with *Eco*RV and *Hin*dIII, and were hybridized with the probe indicated; D, The growth of three independent *nfu1* mutants in media containing different carbon sources is shown. Ten-fold serial dilutions of cells (starting at 10^5 cells) were spotted onto the plates and incubated at 30°C for 2 days. YPD, yeast extract peptone destrose; YPEG, yeast extract peptone ethanol glycerol.

included in this study.

Growth of the *nfu1* mutants was first compared with

that of the wild-type parental strain. Three independent mutants were viable but showed marked growth defects in the medium containing glucose as a carbon source, suggesting that Nfu1 plays an essential role in the physiology of *C. neoformans*. Similar growth defects were observed when the mutants grew in medium containing non-fermentable carbon sources glycerol and ethanol (Fig. 1D). These reduced growth phenotypes of the *C. neoformans nfu1* mutants differed from the phenotype of the *S. cerevisiae nfu1* mutant, which showed wild-type level growth in medium containing either a fermentable or non-fermentable carbon source. In *S. cerevisiae*, only double deletion of *NFU1* and *ISU1* caused considerable growth retardation when the cells were grown in medium containing a non-fermentable carbon source [16]. These previous observations and our current data imply that the role of Nfu1 in *C. neoformans* differs from that of Nfu1 in *S. cerevisiae*.

Mitochondrial Fe-S cluster synthesis is known to contribute to iron homeostasis. Therefore, we determined iron levels in the *nfu1* mutants and compared them with that of the wild type to find if Nfu1 influences iron homeostasis in *C. neoformans*. Intracellular concentrations of other essential metals such as zinc, manganese and copper were also measured because substantial evidence has suggested that iron uptake and metabolism are linked with other essential metals in fungi [23-25]. The results from the analysis using inductively coupled plasma-atomic emission spectroscopy showed that the intracellular concentration of iron, manganese and copper were reduced in the *nfu1* mutant. Interestingly, however, zinc concentration in the *nfu1* mutant was unchanged compared to the wild-type cells (Table 1). These data suggested that while Nfu1 influences not only homeostasis of iron, manganese and copper, zinc homeostasis is independent of Nfu1 function.

Table 1. Metal content in the *nfu1* mutant

	Content (atom/cell, $\times 10^7$)			
	Fe	Zn	Mn	Cu
WT	85.0 ± 1.75[a]	288.0 ± 5.85	5.59 ± 1.5	3.03 ± 0.55
*nfu1*D	76.0 ± 1.09	277.6 ± 4.06	3.62 ± 0.08	2.18 ± 0.44
Reduction (%)[b]	10.59	3.45	42.86	26.67

[a]All values (atoms per cell) were obtained from three biological replicates and are expressed with standard deviations.
[b]Percentage reduction from the wild type.

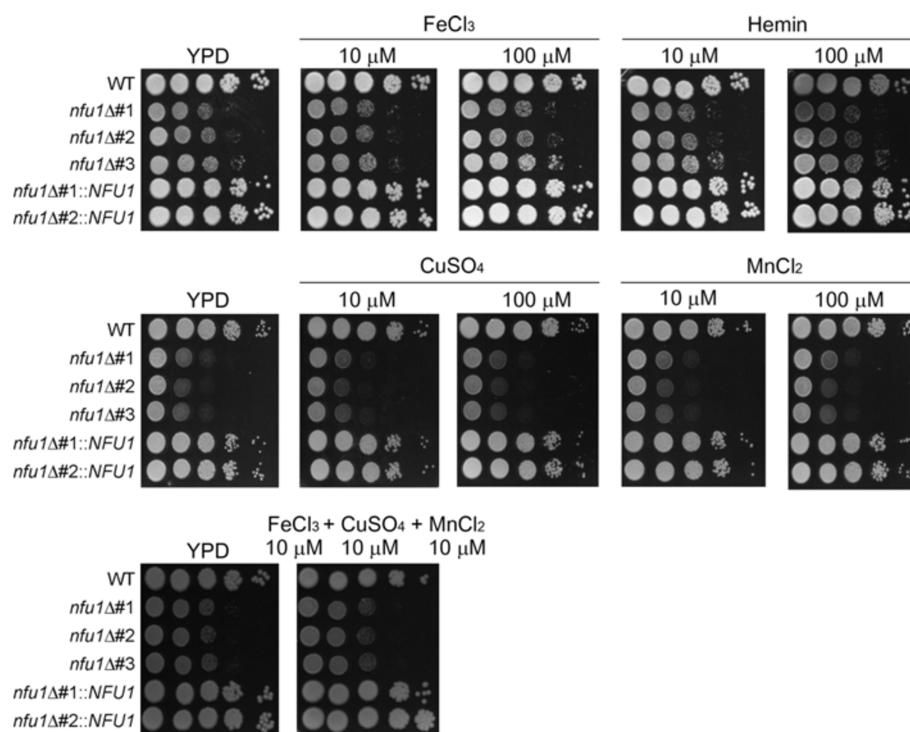

Fig. 2. Reduced growth of the *nfu1* mutants was not restored by metal supplementation. The growth of three independent *nfu1* mutants in media containing different essential metals is shown. Hemin was also added as an alternative iron source. Ten-fold serial dilutions of cells (starting at 10^5 cells) were spotted onto the plates and incubated at 30°C for 2 days. YPD, yeast extract peptone destrose.

Failure in intracellular homeostasis of iron, copper and manganese causes a detrimental effect in the physiology of fungal cells. Therefore, we attributed the growth defects of the *nfu1* mutant to the reduced cellular levels of essential metals and investigated if supplementation of each metal restored the growth of the *nfu1* mutant. As shown in Fig. 2, supplementation with individual metal did not restore the growth of the *nfu1* mutant. Moreover, addition of iron together with copper and manganese also did not support growth of the *nfu1* mutant. These results suggested that growth deficiency of the mutants was caused not only by reduced levels of essential metals but also by other defects such as dysfunctional mitochondrial Fe-S assembly in the cell caused by *NFU1* deletion. Mitochondrial functions are of particular interest in fungal pathogens, including *C. neoformans*. Considerable evidence implies mitochondrial functions in essential metabolic functions such as respiration and lipid biosynthesis, as well as in susceptibility to antifungal drugs [26]. Moreover, the contribution of mitochondrial functions to virulence in the closely related species *Cryptococcus gattii* has been described [26-28].

In this study, we identified the ortholog of *S. cerevisiae* Nfu1, which is involved in the assembly of mitochondrial Fe-S clusters and intracellular iron homeostasis in *C. neoformans*. Nfu1 localized in mitochondria and influenced the homeostasis of essential metals such as iron, copper and manganese. Unlike *S. cerevisiae*, significant growth defects were observed in the mutant lacking *NFU1*. Overall, our results suggest that Nfu1 plays essential roles in Fe-S cluster biosynthesis and intracellular metal homeostasis in *C. neoformans*.

ACKNOWLEDGEMENTS

This study was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT, and Future Planning NRF-2013R1A1A1A05007037.

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