

## Novel subfamily of mitochondrial HMG box-containing proteins: functional analysis of Gcf1p from *Candida albicans*

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Mitochondria of eukaryotic organisms contain populations of DNA molecules that are packed into higher-order structures called mitochondrial nucleoids (mt-nucleoids). In *Saccharomyces cerevisiae*, the compaction of mitochondrial DNA (mtDNA) into mt-nucleoids is mediated primarily by the high-mobility group (HMG) box-containing protein Abf2, which is an important player in stabilization and metabolism of mtDNA. Although it is evident that analogous proteins must exist in other yeast species, an apparently fast divergence rate has precluded their identification, characterization and comparative analysis. Using *in silico* analysis of the complete genome sequence of the pathogenic yeast *Candida albicans* we predicted that the ORF 19.400/19.8030 assigned as *GCF1* encodes a putative mitochondrial HMG box-containing protein. In contrast to Abf2p, which contains two HMG boxes, Gcf1p contains only one C-terminal HMG box. In addition, it contains one putative coiled-coil domain with a potential role in protein dimerization. Fluorescence microscopy analysis of a C-terminally tagged Gcf1p with green fluorescent protein (GFP) revealed its mitochondrial localization in both heterologous (*S. cerevisiae*) and native (*C. albicans*) hosts. Biochemical analyses of DNA-binding properties indicate that Gcf1p is, similarly to Abf2p, a non-specific DNA-binding protein. To analyse the role of Gcf1p in mtDNA metabolism, we constructed strains lacking one functional allele of the *GCF1* gene and carrying one *GCF1* allele under the control of the *MET3* promoter. Under repressible conditions this strain exhibited a more than 3000-fold decrease in levels of *GCF1* mRNA, which was correlated with a substantial decrease in the number of mtDNA copies as well as recombination intermediates. The dramatic effect of reduced levels of Gcf1p on mtDNA metabolism indicates that the protein is involved in essential molecular transactions that relate to the mitochondrial genome.

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**Abbreviations:** AGE, agarose gel electrophoresis; DAPI, 4',6-diamidino-2-phenylindole; EMSA, electrophoretic mobility shift assay; GST, glutathione S-transferase; HMG, high-mobility group; mtDNA, mitochondrial DNA; mtHMG, mitochondrial HMG box-containing protein; mt-nucleoid, mitochondrial nucleoid; qRT-PCR, quantitative real-time RT-PCR.

A supplementary figure, showing phylogenetic trees of mitochondrial HMG box-containing proteins and the superfamily of HMG box-containing proteins encoded by yeast genomes, and a supplementary table of complementation of the *S. cerevisiae*  $\Delta abf2$  mutant by *Candida parapsilosis* *GCF1*, with associated supplementary methods, are available with the online version of this paper.

## INTRODUCTION

Similarly to its nuclear counterpart, mitochondrial DNA (mtDNA) is bound by a variety of proteins with different roles in stabilization, maintenance and inheritance of the genome (Kuroiwa, 1982). It is now generally accepted that the resulting DNA–protein structures [mitochondrial nucleoids (mt-nucleoids)] are fundamental segregating units of mtDNA (Okamoto *et al.*, 1998; Zelenaya-Troitskaya *et al.*, 1998; MacAlpine *et al.*, 2000), and investigations of their structure and dynamics are essential for understanding the principles that govern mitochondrial inheritance (Chen & Butow, 2005; Kucej & Butow, 2007).

In *Saccharomyces cerevisiae*, the major mt-nucleoid component is the non-histone protein Abf2p (Caron *et al.*,

1979; Diffley & Stillman, 1991, 1992). Abf2-like proteins, found in several yeast species (Miyakawa *et al.*, 1996, 2000; Umezaki & Miyakawa, 2002), are tightly bound to mt-nucleoids and exhibit a high affinity for dsDNA (Miyakawa *et al.*, 1995). *In vitro*, Abf2p bends the DNA backbone, resulting in compaction of dsDNA molecules into 190 nm structures resembling the mt-nucleoids (Brewer *et al.*, 2003). The Abf2 protein belongs to the high-mobility-group (HMG) box superfamily containing two tandem HMG boxes responsible for the non-sequence-specific binding of Abf2p to the minor groove of DNA. Similarly to other members of the HMG box-containing protein family, Abf2p exhibits affinity for distorted DNA (e.g. Holliday junctions, DNA cruciforms, DNA bulges and crossovers in supercoiled DNA) and induces looping of linear DNA molecules (Diffley & Stillman, 1991, 1992; Brewer *et al.*, 2003; Friddle *et al.*, 2004). *In vivo*, Abf2p is essential for stabilization of mtDNA in cells grown on a fermentable carbon source such as glucose (Diffley & Stillman, 1991). In addition, it has been shown that Abf2p influences the level of yeast mtDNA recombination intermediates (MacAlpine *et al.*, 1998) and is involved in remodelling of mt-nucleoids in response to changes in mitochondrial metabolism (Kucej *et al.*, 2008).

The involvement of Abf2p in stabilization of *S. cerevisiae* mtDNA indicates that this HMG box-containing protein is an important component of the budding yeast mt-nucleoid. However, extensive searches through databases have not led to identification of an Abf2p homologue, even in relatively closely related yeast species (Nosek *et al.*, 2006). The ability of otherwise unrelated HMG box-containing proteins such as bacterial protein HU, human mitochondrial transcriptional activator mtTF1/TFAM and mitochondrially targeted non-histone protein NHP6A to functionally replace Abf2p both *in vivo* and *in vitro* (Parisi *et al.*, 1993; Kao *et al.*, 1993; Megraw & Chae, 1993) suggests that the presence of the HMG box is sufficient for stabilization of mtDNA. Therefore, it is evident that in different phylogenetic branches the roles of Abf2p have been adopted by unrelated HMG box-containing proteins.

In *Podospora anserina*, the mitochondrial HMG-like protein mthmg1 has been shown to protect mtDNA from deletions (Dequard-Chablat & Allandt, 2002). With the exception of two HMG boxes, mthmg1 does not display significant homology with any mitochondrial HMG box-containing protein (mtHMG). The repertoire of currently known mtDNA packaging proteins was recently expanded by a protein named Glom, purified from the mt-nucleoids of the slime mould *Physarum polycephalum*. Glom has a lysine-rich region with a proline-rich domain in the N-terminal half and two HMG boxes in the C-terminal half (Sasaki *et al.*, 2003). Again, with the exception of HMG boxes, Glom does not exhibit homology with Abf2p.

Recently, we have performed a comparative analysis of homologues of the components of mt-nucleoids, especially proteins containing HMG boxes fulfilling the role(s) of

Abf2p (Nosek *et al.*, 2006). We were able to identify Abf2p homologues only in species closely related to *Saccharomyces* spp. Even in these cases, homology between the corresponding proteins is low (e.g. 28.5% identity between *S. cerevisiae* and *Kluyveromyces lactis* Abf2p) compared with that of other mt-nucleoid proteins (Nosek *et al.*, 2006). Analysis of available genome sequences does not reveal a clear sequence homologue of Abf2p in species below the *Ashbya gossypii* branch on the phylogenetic tree, indicating that species such as *Candida albicans*, *Candida parapsilosis*, *Debaryomyces hansenii*, *Yarrowia lipolytica* and *Schizosaccharomyces pombe* employ alternative modes of mtDNA packaging. This divergence might be caused by dramatic alterations in the Abf2p sequence without affecting its function. According to an alternative possibility, another mtHMG might have been recruited to ensure mtDNA packaging. Indeed, in *C. albicans* and *D. hansenii* we have found ORFs encoding proteins [EAK92709 in *C. albicans* (CaO19.400, CaO19.8030; *GCF1*); CAG90643 in *D. hansenii* (DEHA0G15059 g)] containing at least one HMG box, one coiled-coil domain and a putative mitochondrial N-terminal leader sequence (Nosek *et al.*, 2006). There are two interesting features exhibited by both proteins: (i) their lengths (245 aa in *CaGcf1p*; 275 aa in *DhGcf1*) are similar to that of the human mtTF1 (246 aa), and (ii) they are relatively lysine-rich, analogously to the N-terminal domain of the mtDNA packaging protein (Glom) of the slime mould (Sasaki *et al.*, 2003). SMART analysis revealed that, in contrast to Abf2p, mtTF1, mthmg1, Glom and *DhGcf1p*, *CaGcf1p* contains only one HMG box, indicating that in *C. albicans* a single HMG box is sufficient for mtDNA packaging. *Gcf1* protein thus seems to represent a member of a novel class of mtDNA packaging proteins, and therefore we decided to pursue a study of its role in the maintenance of *C. albicans* mtDNA. Importantly, a homologue of *Gcf1p* has been subsequently identified biochemically as a component of the mt-nucleoid of *C. parapsilosis* (Miyakawa *et al.*, 2009).

In this work we performed *in silico* analysis of mtHMGs and analysed biochemical properties and physiological functions of the *Gcf1* protein in *C. albicans*. Using green fluorescent protein (GFP) fused to the C terminus of *Gcf1p* we demonstrated that it is targeted to mitochondria of both its native (*C. albicans*) and heterologous (*S. cerevisiae*) hosts. We have analysed DNA-binding properties of *Gcf1p* using its recombinant version expressed in *Escherichia coli*. We tested the ability of *GCF1* to complement  $\Delta abf2$  mutation in *S. cerevisiae*. Finally, we analysed the consequences of deletion of *GCF1* in *C. albicans* on mtDNA metabolism in this pathogenic yeast.

## METHODS

**Yeast strains.** *C. albicans* CBS562<sup>NT</sup> was provided by Dr H. Fukuhara (Institut Curie, Orsay, France). *C. albicans* CA14 (*ura3:: $\lambda$ imm434/ura3:: $\lambda$ imm434*) was provided by Dr A. J. Mitchell

(Columbia University, New York, USA). *S. cerevisiae* strain GG595 (*MATa/MAT $\alpha$  ura3<sup>-</sup>/ura3<sup>-</sup>*) was obtained from dr. H. Y. Steensma (Leiden University, The Netherlands), and strain YAM 101 (*MATa/MAT $\alpha$   $\Delta$ abf2::TRP1/ABF2 ade2-1/ade2-1 ura3-1/ura3-1 his3-11,15/his3-11,15 trp1-1/trp1-1 leu2-3, 112/leu2-3,112 can1-100/can1-100*) was provided by Dr D. A. Clayton (Howard Hughes Medical Institute, Chevy Chase, USA). The haploid strains used for complementation experiments YAM 101-1a (*MATa ABF2 ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*) and YAM 101-1b (*MATa  $\Delta$ abf2::TRP1 ade2-1 ura3-1 his3-11,15 leu2-3,112 can1-100*) were prepared by tetrad dissection of the strain YAM 101. Yeast cultures were grown in YPD medium [1% (w/v) yeast extract (Difco), 2% (w/v) Bacto Peptone (Difco), 2% (w/v) glucose] at 28 °C. In the case of *C. albicans*, media were supplemented with 80  $\mu$ g uridine ml<sup>-1</sup>. Synthetic medium was 0.17% (w/v) Yeast Nitrogen Base without amino acids and ammonium sulfate (Difco), 0.5% (w/v) ammonium sulfate (Lachema) containing 2% (w/v) glucose (SD), 3% (v/v) glycerol (SG) or 2% (w/v) galactose (SGal). Transformation of *S. cerevisiae* was done as described by Gietz & Schiestl (1995). Electroporation of *C. albicans* was performed according to the protocol of De Backer *et al.* (1999).

**DNA manipulations.** The oligonucleotides listed in Table 1 were synthesized by Invitrogen or Sigma-Aldrich. PCRs (20–50  $\mu$ l) were performed using 1 U Vent DNA polymerase (New England Biolabs) or *Taq* DNA polymerase (Invitrogen) and contained each dNTP at 200  $\mu$ M, corresponding primers at 1  $\mu$ M and 100 ng genomic DNA or 1 ng plasmid DNA. PCR fragments were purified from agarose gels using a Qiagen Gel Extraction kit. Southern blot analysis was performed as described by Sambrook & Russell (2001). DNA-modifying enzymes were used according to the instructions provided by the corresponding suppliers.

**Construction of plasmids.** For expression in *E. coli*, *GCF1* amplified from genomic DNA using primers CA\_HMG\_F+START and CA\_HMG\_R+STOP was cloned into pGEX-2T (Amersham-Pharmacia) linearized with *Eco*RI and blunt-ended with Klenow fragment of DNA polymerase I. For *in vivo* localization in *S. cerevisiae*, the PCR fragment obtained by amplification of *GCF1* using primers CA\_HMG\_F+START and CA\_HMG\_R was ligated into pUG35 (Niedenthal *et al.*, 1996; provided by Dr J. H. Hegemann, Heinrich-Heine-Universität, Düsseldorf, Germany) linearized with *Sma*I. For *in vivo* localization in *C. albicans*, *GCF1* was PCR-amplified using primers 5BamORF and RSp3ORF and digested with *Bam*HI and *Esp*3I. The ORF encoding an enhanced version 3 of yeast GFP (yEGFP3) was PCR-amplified using the primers 5GFPEsp3 and 3PstGFP and digested with *Pst*I and *Esp*3I. Both PCR products were ligated into the pCaExp vector (kindly provided Dr P. E. Sudbery, Sheffield University, UK) digested with *Bam*HI and *Pst*I. For complementation experiments in *S. cerevisiae*, *GCF1* was PCR-amplified using primers CA\_HMG\_F+START and CA\_HMG\_R+STOP, and ligated into pYES2/CT (Invitrogen) linearized with *Eco*RI and blunt-ended with Klenow fragment of DNA polymerase I. All plasmid constructs were verified by restriction enzyme mapping as well as by DNA sequence analysis.

**Complementation of the  $\Delta$ abf2 mutant of *S. cerevisiae* by *GCF1*.** Vectors pYES2/CT and pYES2CT-*GCF1* were transformed into *S. cerevisiae* strains *ABF2* and  $\Delta$ *abf2* and cells were plated on minimal glycerol medium. Cells from individual clones were inoculated into 2 ml minimal glycerol medium and cultivated for 24 h at 28 °C. Minimal media containing glucose, glycerol or galactose were inoculated from the pre-culture at a concentration of  $1 \times 10^5$  cells ml<sup>-1</sup>. The cell suspensions were cultivated for 24 h at 28 °C, and cells (200 cells per plate) were spread on minimal glucose and glycerol medium and cultivated for 3 days at 28 °C. Frequency of

standard (grande) and petite colonies was determined by triphenyl tetrazolium chloride (TTC) assay (Ogur *et al.*, 1957).

#### Purification of glutathione S-transferase (GST) fusion protein.

Recombinant GST-Gcf1 protein was purified as either GST fusion or native protein. An overnight bacterial culture (20 ml) was inoculated into 1 l LBA medium [3.2% (w/v) tryptone (FisherBiotech), 2% (w/v) yeast extract (Difco), 0.5% (w/v) NaCl, ampicillin (100  $\mu$ g ml<sup>-1</sup>)] and grown at 37 °C to final OD<sub>600</sub> 0.7. The culture was then induced for 3 h at 30 °C with 1 mM IPTG (Sigma). Cells were washed once with ice-cold double-distilled water, resuspended in 20 ml buffer G [20 mM Tris/HCl, pH 7.4, 100 mM NaCl, 0.1% Triton X-100, 10 mM 2-mercaptoethanol, protease inhibitor mixture (Complete, Boehringer Mannheim), 50 U DNase I (Promega)], sonicated, and centrifuged at 25 000 g for 30 min at 4 °C to remove insoluble material. The supernatant was mixed with 0.5 ml glutathione-agarose (Sigma) prewashed with buffer G, and incubated for 60 min on ice with occasional mixing. Beads were loaded onto a 5 ml column, and washed with 100 ml buffer G (without protease inhibitor mixture) followed by 50 ml ice-cold solution A (20 mM Tris/HCl, pH 7.4, 100 mM NaCl). The washed beads were resuspended in 1 ml solution A [supplemented with 2.5 mM CaCl<sub>2</sub> and RNase A (75  $\mu$ g ml<sup>-1</sup>)] containing thrombin (0.05 U  $\mu$ l<sup>-1</sup>, Amersham Biosciences) and incubated overnight at 4 °C. The cleaved Gcf1p was eluted five times in 1.0 ml solution A and stored at -80 °C. GST-Gcf1 fusion protein and GST were eluted from the column by 50 mM glutathione in solution A. Fractions were assayed for purity by SDS-PAGE (Laemmli, 1970) and stained with Coomassie Brilliant Blue.

**DNA-binding assays *in vitro*.** Gel retardation assays were performed as described by Sasaki *et al.* (2003). In brief, purified GST-Gcf1 or Gcf1 protein (20–200 ng) was mixed with *Nde*I-digested *C. albicans* mtDNA (purified as described by Defontaine *et al.*, 1991). The samples were incubated for 1 h in DNA-binding buffer (20 mM Tris/HCl, pH 7.4, 1 mM EDTA, 50 mM glutathione) at room temperature and then loaded onto a 0.8% (w/v) agarose gel. DNA fragments and DNA-protein complexes were separated at 5–10 V cm<sup>-1</sup> for 45–60 min in 45 mM Tris-borate, 1 mM EDTA (0.5  $\times$  TBE) buffer. After electrophoresis, the agarose gels were stained for 20 min with ethidium bromide (0.5  $\mu$ g ml<sup>-1</sup>) followed by 10 min destaining in distilled water. For radioactive electrophoretic mobility shift assays (EMSA) (Fig. 3d), *C. albicans* mtDNA was digested with a combination of *Bam*HI and *Pvu*II and dephosphorylated with shrimp alkaline phosphatase, and the DNA fragments were labelled by T4 polynucleotide kinase using [ $\gamma$ -<sup>32</sup>P]ATP as a substrate. DNA-binding assays were performed as described above. Nonlabelled mtDNA digested with a combination of *Bam*HI and *Pvu*II was used as a DNA competitor.

#### Construction of a strain with a conditional allele of *GCF1*.

For the disruption of one allele, *GCF1*, 400 bp from the gene sequence was PCR-amplified using primers 5BamORF and 3PstORF, digested with *Bam*HI and *Pst*I, and ligated into the plasmid pCaDis (Care *et al.*, 1999; the plasmid was kindly provided by Dr P. E. Sudbery, Sheffield University, UK) digested with the same restriction enzymes. The ligation mixture was transformed into *E. coli* DH5 $\alpha$  and the plasmids were verified by restriction enzyme analysis. The resulting plasmid pCaDis*GCF1* was digested with *Bst*BI approximately in the middle of the inserted 400 bp and transformed into *C. albicans* strain CAI4 by electroporation. Homologous recombination yielded the *GCF1* gene under control of the *MET3* promoter (Fig. 4a). Transformants were verified by both PCR and Southern blot analyses, and the resulting strain was designated *GCF1/P<sub>MET3</sub>-GCF1*.

For disruption of the second allele of *GCF1*, the sequences (1000 bp) flanking the *GCF1* ORF were PCR-amplified using primers R5orf and D5OUT (5' end) and R3OUT and D3orf (3' end), and after digestion

**Table 1.** Oligonucleotides used in this study

Name	Sequence	Application*
CA_HMG_F + START	ATGTTGAGATCATTTGTAACATC (23-mer)	1, 3
CA_HMG_R	AAAGTCATCCTCCACTTTGTAG (22-mer)	1, 4
CA_HMG_R + STOP	TCAAAAAGTCATCCTCCACTTTG (22-mer)	1
5BamORF	ATGGATCCATGTTGAGATCATTTGTAACA (29-mer)	1, 2
REsp3ORF	ATCGTCTCTACATAAAAGTCATCCTCCACTTTGTA (34-mer)	1
5GFPEsp3	ATCGTCTCAATGTCTAAAGGTGAAGAATTA (30-mer)	1, 3
3PstGFP	ATCTGCAGTTATTTGTACAATTCATCCAT (29-mer)	1
3PstORF	ATCTGCAGGTCAGTAATTCACCTTTCCGG (29-mer)	1, 3, 4
R3OUT	ATGAGCTCTATTCTCGAATCAGTTGATT (29-mer)	1, 2, 3
D3orf	ATTGCGGCCGCCCTCAATCAACTAAAAGAGGTC (32-mer)	1
R5orf	ATACTCGAGAGATGAACAATAAAATTGAC (30-mer)	1
D5OUT	ATGGGCCCACTAATATTTTACCTGCAGGG (29-mer)	1
pMET	ATGAAAAGACAAGAGAGC (18-mer)	4
GFP	AGATGGTGATGTTAATGG (18-mer)	4
pGEX5	GGCAAGCCACGTTTGGTG (18-mer)	4
pGEX3	GGAGCTGCATGTGTCAGAGG (20-mer)	4
5ORFterm	ATCTGCAGAGAACTCAATCAACTAAA (27-mer)	3
D3OUT	ATTGCGGCCGCCGATGGATGGATGACGAGAAC (32-mer)	2
IMH3OUT	AGAGGTATGGGTTCCATCGAT (21-mer)	3
3PstKO	ATCTGCAGTGCCTTCTCTTCCTCGGGGAA (29-mer)	2
CaAbf2Rev	TCAATAGTCAAATAAAGTAGC (21-mer)	3
CaUra3in3	TAATTGGCCAGTCTTTTCAAATAAGC (27-mer)	2
CamtHMG1in5	GTGGTACCATCACCGACTTCA (21-mer)	3
CamtHMG1in3	ATCCGCAGCAACCGTCAATT (20-mer)	3
MPAout3	AGATCCACTAGTCTAGAGCGGCC (24-mer)	3
MPAout5	CTAGAAAAGTATAGGAACTTCCTCGAG (26-mer)	3
D5R5in	ATATTCTCCATTATACTTTGTATTGG (26-mer)	2
D3R3in	AACTATGGTCAAAAAGGTCTAAAAA (25-mer)	2
CanEco31	ACGTGGTCTCATGGAATTAACACCCACACAG (31-mer)	2
Can3CBam	GCGGATCCTAAATTAGTCGACTAGT (25-mer)	2
5RTcaAct1	TGTCTTTGTA CTCTTCTGGTAGAAC (25-mer)	5
3RTcaAct1	CTAGTAGTGAACTGTAACCACGTT (25-mer)	5
5RTcaGCF1	CAAAAAGCTATTTTACTCCTAAACCA (25-mer)	5
3RTcaGCF1	ATCTTCTTTACTGATTTGGTATTGC (25-mer)	5
5RTcaNAD2	TTCAAAATGGGATTAGCACCTT (22-mer)	5
3RTcaNAD2	TGTATATGCTGCCACGGCTA (20-mer)	5

\*1, PCR amplification for cloning; 2, PCR amplification to prepare probe for Southern blot hybridization; 3, PCR verification; 4, sequencing; 5, qRT-PCR.

with *XhoI* and *ApaI* (5' end) and *SacI* and *NotI* (3' end) were ligated into the vector pSFI (provided by Dr J. Morschhäuser, Universität Würzburg, Germany) containing an *MPA<sup>R</sup>-FLP* cassette (Wirsching *et al.*, 2000). The cassette enables selection of transformants based on their resistance to mycophenolic acid (MPA; final concentration 10 µg ml<sup>-1</sup> in synthetic media) and removal of *MPA<sup>R</sup>* by expression of flip-flop (FLP) recombinase driven from the *SAP2* promoter (Wirsching *et al.*, 2000; Fig. 4a). The ligation mixture was transformed into *E. coli* DH5 $\alpha$  and the resulting plasmids were verified by restriction enzyme analysis. The *MPA<sup>R</sup>-FLP* cassette containing flanking sequences was cut out of the plasmid with *ApaI* and *SacI* and used for electroporation of *C. albicans* strain *GCF1/P<sub>MET3</sub>-GCF1* (see above). The transformants were verified by PCR and Southern blot analyses, and the strain carrying one conditional allele of *GCF1* and lacking the second allele of *GCF1* was named  $\Delta gcf1/P_{MET3}-GCF1$ .

**mtDNA purification.** *C. albicans* strains were grown to OD<sub>600</sub>=1.5 as described above. Mitochondria were purified according to Daum *et al.* (1982) with slight modifications. In brief, cells were harvested, washed once with H<sub>2</sub>O and once with 1.2 M sorbitol, and resuspended in Zymolase-buffer (1.2 M sorbitol, 20 mM KP<sub>i</sub> buffer, pH 7.4, to 1 ml per 0.15 g wet weight cells). Zymolase 20T was added to 8 mg per g of cells (wet weight) and incubated at 30 °C for 10–15 min until conversion of cells to spheroplasts. After centrifugation, spheroplasts were resuspended in homogenization buffer [10 mM Tris/HCl, pH 7.4, 1 mM EDTA, 0.6 M sorbitol, 0.2% (w/v) BSA, 1 mM PMSF] and lysed by 20 strokes in a tight fitting Dounce homogenizer. Lysates were centrifuged twice at 3500 r.p.m. in an SS34 rotor (Sorvall) for 5 min at 4 °C, and the resulting supernatant once at 9000 r.p.m. in an SS34 rotor for 15 min. The crude mitochondrial pellet was resuspended in lysis buffer (50 mM

EDTA, 10 mM Tris/HCl, pH 7.4, 75 mM NaCl). SDS was added to a final concentration of 1% (w/v) and the mixture was kept on ice for 10 min. Subsequently, proteinase K was added to a final concentration of 0.5 mg ml<sup>-1</sup>, and the samples were incubated at 37 °C for 30 min. Lysates were extracted with phenol:chloroform (1:1), DNA was ethanol-precipitated, washed and dried, and finally resuspended in T<sub>10</sub>E<sub>1</sub> buffer (10 mM Tris/HCl, pH 7.4, 1 mM EDTA), aliquoted and stored at -70 °C until use. Samples (10 µg) of mtDNA were subjected to restriction enzyme digestions according to the manufacturer's protocols (Fermentas) and separated by 2D agarose gel electrophoresis (2D-AGE).

**2D-AGE, hybridization and detection of mtDNA recombination and replication intermediates.** Neutral 2D-AGE was carried out as described elsewhere (Brewer & Fangman, 1987). Fragments between 5.5 and 11 kb were separated on 0.3% (w/v) 1 × TBE-agarose gels at 0.9 V cm<sup>-1</sup> and room temperature for 24 h in the first dimension, followed by separation on 0.8% (w/v) 1 × TBE-agarose gels containing ethidium bromide (300 ng ml<sup>-1</sup>) at 3 V cm<sup>-1</sup> and 4 °C for 15 h in the second dimension. Southern blots of 2D gels were hybridized with specific PCR probes of *C. albicans* mtDNA as indicated (Table 1) for 3–4 h at 65 °C in 7% (w/v) SDS, 250 mM NaHPO<sub>4</sub> (pH 7.2), 1 mM EDTA and 0.1% (w/v) BSA (Church & Gilbert, 1984). Blots were then washed twice for 15 min at 65 °C with wash buffer [5% (w/v) SDS, 40 mM NaHPO<sub>4</sub> (pH 7.2), 1 mM EDTA]. Filters were exposed to X-ray films for 1–14 days or to Storage Phosphor Screens (GE Healthcare) for 12–72 h. Signal detection of screens was performed with a Typhoon Trio Phosphor Imager (GE Healthcare).

**Purification of total cellular DNA and RNA and quantitative real-time RT-PCR (qRT-PCR).** *C. albicans* strains were grown as described above and total DNA was isolated according to our standard procedure (Sedman *et al.*, 2005). Total RNA was purified from *C. albicans* strains as follows. Cultures (3 ml) were grown to mid-exponential phase as described above, harvested and resuspended in 100 µl ice-cold RNA-buffer (50 mM Tris/HCl, pH 7.4, 100 mM NaCl, 10 mM EDTA). Glass beads (50 µl) were added and the samples were vortexed at maximum speed for 3 min at 4 °C. RNA-buffer [450 µl containing 1% (w/v) SDS] and 450 ml equilibrated phenol were added, and the samples were vortexed as described above followed by centrifugation for 10 min at 4 °C, precipitation of RNA with an equal volume of 2-propanol for 60 min at room temperature, sedimentation of RNA by centrifugation and resuspension of the resulting RNA in RNase-free water. Total RNA samples (5 µg) were treated with DNase I (Fermentas), and 1 µg of the resulting RNA was used in reverse transcriptase reactions according to the supplier's protocol (Fermentas). cDNA (50 ng) or total cellular DNA (50 ng), were analysed by qRT-PCR using the AB 7500 Real-Time PCR System (Applied Biosystems; conditions: 40 cycles; 95 °C 20 s, 60 °C 30 s, 72 °C 33 s). Maxima SYBR Green qPCR Master Mix (Fermentas) was used to amplify a 170 bp portion of *GCF1*, *CaACT1* or *CaNAD2* using RT-PCR primers according to Table 1. For all independent RNA preparations, DNase I-treated RNA without reverse transcriptase was included as a control in qRT-PCR experiments to exclude false-positive results due to DNA contamination (Maxima standard protocol, Fermentas). All experiments were performed on four biological replicates for each strain and were analysed in triplicates.

**Fluorescence microscopy.** *S. cerevisiae* strain GG595 was transformed with pUG35-*GCF1* or pUG35, and cells of *C. albicans* CA14 were transformed with p*GCF1*-GFP digested with *StuI* (in the *RP10* gene), allowing its insertion into the genome. Cells were cultivated in synthetic glucose medium for 24 h at 28 °C. The cells were centrifuged at 3000 g for 5 min, and washed three times with sterile distilled water. 4',6-diamidino-2-phenylindole (DAPI) was added to a

final concentration of 1 µg ml<sup>-1</sup> and the cell suspension was incubated for 1 h at room temperature. The cells were visualized by an Olympus BX50 microscope equipped with a DP70 camera (Olympus Optical) and an appropriate filter set.

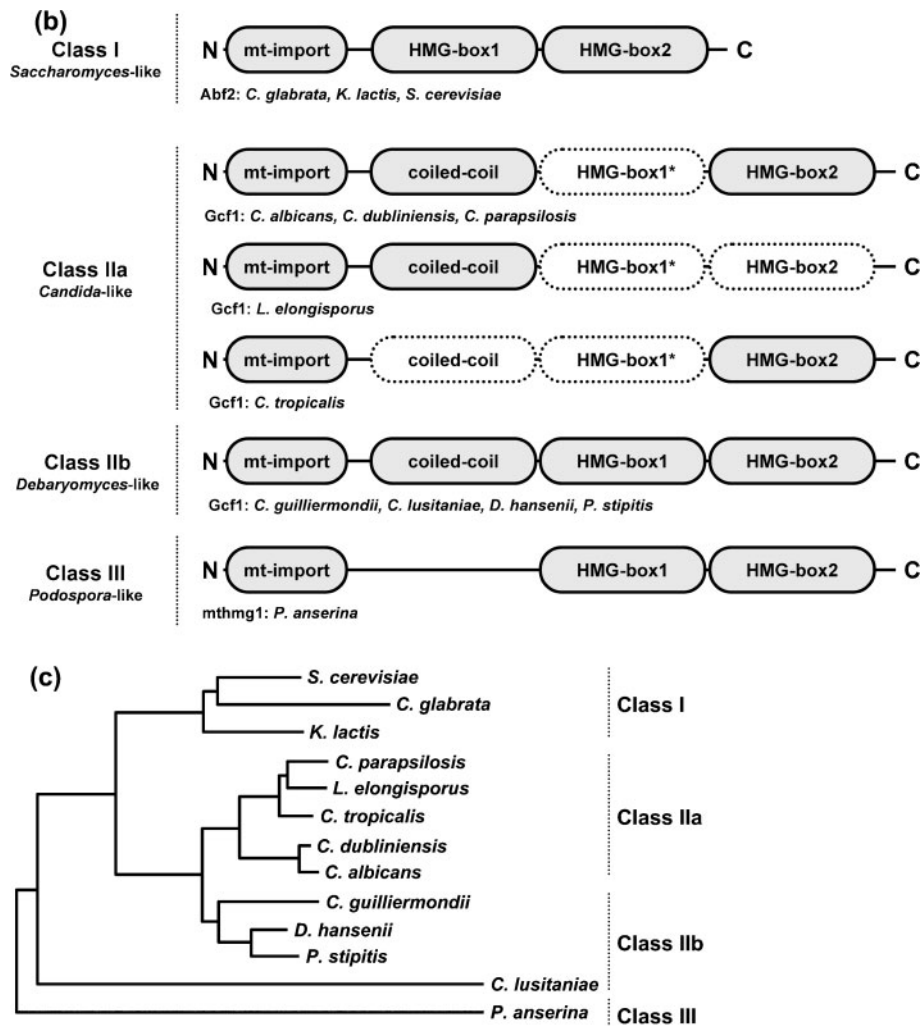
**In silico analyses.** Homologues of *CaGcf1* protein (orf19.400/orf19.8030) were identified in the *Candida* database of the Broad Institute ([http://www.broad.mit.edu/annotation/genome/candida\\_group/MultiHome.html](http://www.broad.mit.edu/annotation/genome/candida_group/MultiHome.html)) using the BLASTP algorithm at default settings (i.e. E value <10<sup>-3</sup>, BLOSUM62 matrix). The query revealed a single hit per genome in *C. albicans*, *Candida guilliermondii*, *Candida lusitanae*, *C. parapsilosis*, *Candida tropicalis*, *D. hansenii* and *Lodderomyces elongisporus*. Similarly, TBlastN searches identified single candidates in assembled genome contigs of *Candida dubliniensis*, *C. parapsilosis* (The Wellcome Trust Sanger Institute; <http://www.sanger.ac.uk/Projects/Fungi/>), and *Pichia stipitis* (DOE Joint Genome Institute; <http://genome.jgi-psf.org/Picst3/Picst3.home.html>). The sequence alignments and comparisons were done using the AlignX utility of the Vector NTI 10.1.1 package (Invitrogen) and the GeneDoc program (Nicholas *et al.*, 1997). Mitochondrial import sequences were detected using MitoProt II (Claros & Vincens, 1996; <http://ihg2.helmholtz-muenchen.de/ihg/mitoprot.html>). The potential to form coiled-coil domains based on the analysis of heptad repeats in three windows of 14, 21 and 28 amino acids was calculated with PCOILS (<http://toolkit.tuebingen.mpg.de/pcoils/>) using the MTIDK matrix. The HMG boxes were predicted using SMART (<http://dylan.embl-heidelberg.de/>; Letunic *et al.*, 2006) and InterProScan (<http://www.ebi.ac.uk/Tools/InterProScan/>). Phylogenetic analyses were performed using the MEGA 4 software package (Tamura *et al.*, 2007).

## RESULTS AND DISCUSSION

### In silico analysis of mtHMGs

With the aim of identifying putative mitochondrial functional homologues of *S. cerevisiae* Abf2 protein in non-*Saccharomyces* species, we first performed an *in silico* analysis of available genomic sequences. We identified a putative mitochondrial HMG box-containing protein encoded by the genome of *D. hansenii* (*DhGcf1p*) and subsequently its homologue in *C. albicans* (*CaGcf1p*) using the sequence of the HMG box of the Abf2 protein of *K. lactis* as a query (Nosek *et al.*, 2006). Following inspection of available genomic sequences, we identified homologous sequences in the genomes of *C. dubliniensis*, *C. guilliermondii*, *C. parapsilosis*, *C. tropicalis*, *L. elongisporus* and *P. stipitis* (Fig. 1a). Importantly, a *Gcf1* homologue was also identified by proteomic analysis of mt-nucleoids in *C. parapsilosis* (Miyakawa *et al.*, 2009). In contrast to their *D. hansenii* counterpart, which contains two putative HMG boxes, *Gcf1p* of *C. albicans* contains only one region with clear HMG sequence motifs. Comparison of amino acid sequences of several yeast mitochondrial HMG box-containing proteins indicates a relatively high rate of evolutionary diversification, and suggests that they can be separated into several families based on the number of putative HMG boxes as well as the presence/absence of a coiled-coil domain supposedly involved in protein dimerization (Fig. 1b). Thus, acquisition of the second HMG box may have been a prerequisite for loss of the coiled-coil





**Fig. 1.** *In silico* analysis of mtHMGs. (a) Amino acid sequence alignment of yeast mtHMGs (class II members). The sequences were aligned using the AlignX utility of the Vector NTI 10.1.1 program package (Invitrogen) and the GeneDoc utility (Nicholas *et al.*, 1997). CANAL, *C. albicans*; CANDU, *C. dubliniensis*; CANGU, *C. guilliermondii*; CANLU, *C. lusitaniae*; CANPA, *C. parapsilosis*; CANTR, *C. tropicalis*; DEBHA, *D. hansenii*; LODEL, *L. elongisporus*; PICST, *P. stipitis*. Positions of coiled-coil domain and HMG boxes in CaGcf1 (CANAL; subclass IIa) and DhGcf1 (DEBHA; subclass IIb) predicted by SMART are indicated above and below the alignment, respectively. Mitochondrial import presequences identified using MitoProtII are underlined. The probabilities of protein import into mitochondria were 0.9939 (CANAL), 0.9976 (CANDU), 0.9378 (CANGU), 0.8854 (CANLU), 0.9673 (CANPA), 0.6683 (CANTR), 0.9763 (DEBHA), 0.9986 (LODEL) and 0.9446 (PICST). (b) Modular structures of fungal mtHMGs. Domains predicted with low confidence are shown with dotted borders. The coiled-coil domain in the N-terminal region of CANAL, CANDU, CANGU, CANLU, CANPA, DEBHA, LODEL and PICST proteins was predicted by PCOILS with probabilities of 0.9–1.0 in at least two heptad repeat frames (i.e. 14, 21 and 28 amino acids). In the *C. tropicalis* homologue the predicted PCOILS probabilities were significantly lower (<0.6, 0.7 and 0.8 for frames 14, 21 and 28, respectively). The HMG boxes (Pfam and SMART accession numbers are PF00505 and SM00398, respectively) were predicted using SMART (normal mode) and InterProScan at default settings. The region corresponding to HMG box 1 is weakly conserved among the members of class II. This domain has not been detected by SMART and InterProScan in any of the subclass IIa proteins. Note that in the case of *L. elongisporus* the prediction of HMG box 2 was below the threshold, but its presence was inferred from the sequence homology. The three classes of fungal mtHMGs were defined using the protein length and the presence of protein domains. (c) Phylogenetic trees of fungal species with known mtDNA packaging proteins. The neighbour-joining tree was calculated from the D1/D2 sequences of the large subunit rRNA gene. The two groups within class II correspond to the split on the phylogenetic tree, which also supports the idea that subclass IIb architecture is ancestral to the subclass I and IIa proteins.

domain in the *S. cerevisiae* group. Based on these data, we suggest naming the group of all mtHMGs the mtHMG superfamily and, based on their modular structure (Fig. 1b), dividing it into four groups, namely class I (*Saccharomyces* Abf2-like), class IIa (*Candida* Gcf1-like), class IIb (*Debaryomyces* Gcf1-like) and class III (*Podospira* mthmg1-like). These subfamilies are consistent with the distribution of the corresponding species on the phylogenetic tree (Fig. 1c). The division of class II into two subclasses is based on the fact that all members of class IIb mtHMGs contain all three modules found in HMG box proteins, while the HMG box 1 was detected neither by SMART nor by the InterProScan utility in any of the class IIa proteins, indicating that this domain rapidly accumulates changes within this region. We suggest that the class IIb members represent an ancestral type of mtHMG.

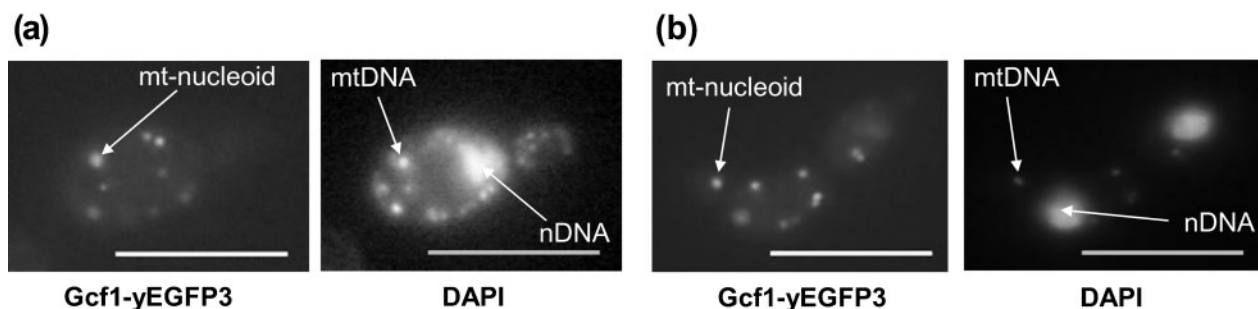
### Gcf1 protein is localized into mitochondria of both *C. albicans* and *S. cerevisiae*

*In silico* analysis of Gcf1 amino acid sequences indicated the presence of an N-terminal mitochondrial targeting sequence (PSORT II and MITOPROT indicated 69.6 and 99.39 % probability of mitochondrial localization, respectively). To verify that the protein is targeted to mitochondria, we constructed two plasmids carrying Gcf1 with C-terminally fused GFP. The first plasmid, pUG35-*GCF1*, was designed for expression of the fusion proteins in *S. cerevisiae*. Fig. 2(a) shows that when expressed in *S. cerevisiae*, Gcf1p targets GFP into mitochondria, while the GFP without Gcf1 at the N terminus is localized in the cytoplasm (data not shown). Similarly, when the Gcf1-GFP fusion protein was expressed in *C. albicans* using the plasmid p*GCF1*-GFP as expression vector, most of the fluorescence was concentrated into the cellular compartments whose co-staining with DAPI indicates that they represent mitochondria (Fig. 2b).

After our *in silico* identification (Nosek *et al.*, 2006), the Gcf1 protein was implicated in regulation of promoter

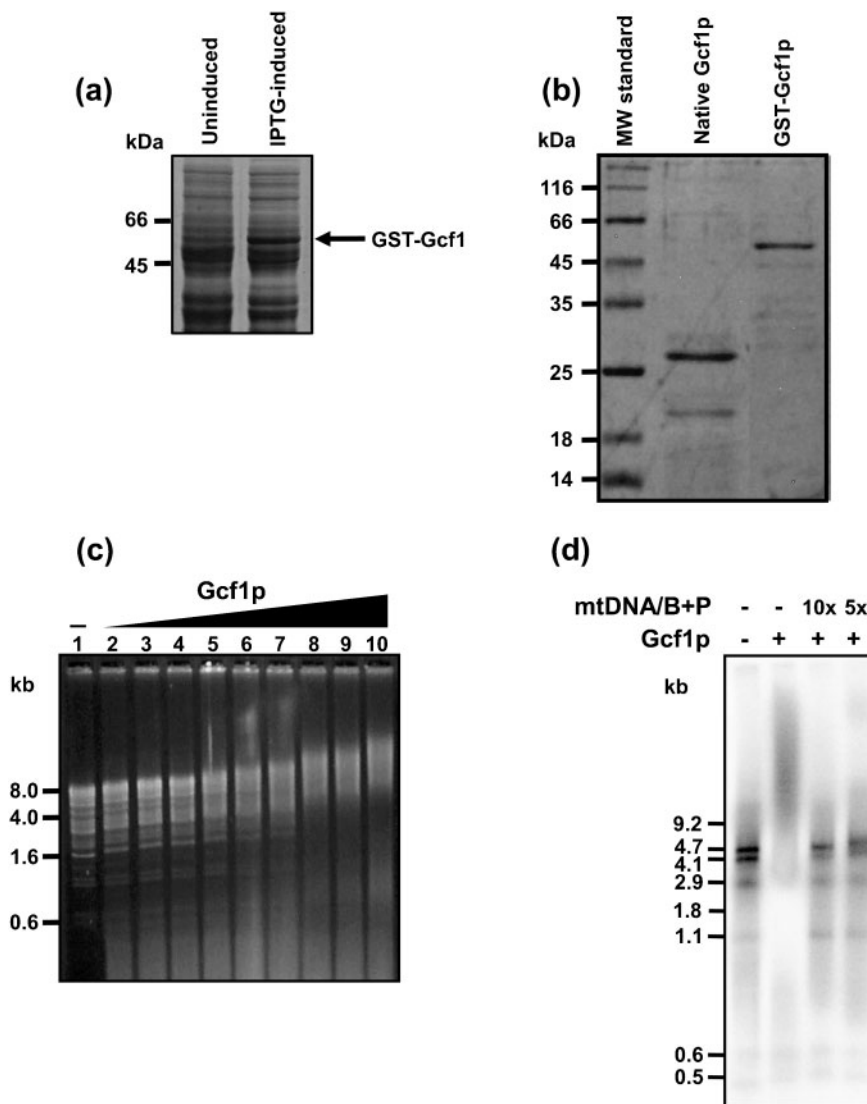
activity of *HWP1*, a gene encoding a hyphae-specific adhesin in *C. albicans* (Kim *et al.*, 2007). There are two possible explanations for this observation. Gcf1p may exhibit dual (mitochondrial and nuclear) localization and thus fulfil two distinct roles in the cell. The nuclear isoform may participate in gene regulation and the mitochondrial isoform in packaging of mtDNA and/or mtDNA metabolism. However, because Kim *et al.* (2007) used crude protein extracts as a source for purification of proteins binding to the promoter region, the binding of Gcf1p to the nuclear sequence might have been artefactual. For example, Gcf1p as a non-specific DNA-binding protein could have been released from mitochondria upon preparation of protein extracts, resulting in its binding to the *Hwp1* promoter *in vitro*. In either case, our experiments clearly demonstrate mitochondrial localization of Gcf1p and thus indicate its role in mtDNA packaging and/or metabolism.

Mitochondrially targeted HMG box proteins seem to be the fastest evolving component of mt-nucleoids (Miyakawa *et al.*, 2009). Therefore, it is difficult to reconstruct the phylogenetic relationships among the various members of the mtHMG family. To circumvent this problem we compared the HMG boxes of selected members of the mtHMG superfamily, including Gcf1, with the HMG boxes of various HMG1-containing proteins, in a similar manner to that reported by Baxeavanis & Landsman (1995). The phylogenetic analysis revealed that the *Ca*Gcf1 HMG box clusters together with the *Cp*Gcf1 HMG1 box and one of the two HMG boxes of the *Dh*Gcf1 protein (DEBHA\_Gcf1.2) (Supplementary Fig. S1). Although some branches of the phylogenetic tree must be taken with caution due to the weak homology, the presence of the second *Dh*Gcf1 HMG box (DEBHA\_Gcf1.1) on a different branch and the relatively distant position of the *Saccharomyces* Abf2-like HMG boxes underline the relatively fast evolution, apparently due to modification of the HMG boxes, in proteins involved in mtDNA packaging.



**Fig. 2.** Gcf1 co-localizes with mtDNA. Co-localization of fusion protein Gcf1-GFP with mtDNA in the cells of *S. cerevisiae* (a) and *C. albicans* (b) visualized by fluorescence microscopy. The cells expressing Gcf1-GFP were stained with DAPI as described in Methods. Bars, 10  $\mu$ m.





**Fig. 3.** Gcf1 is a DNA-binding protein. *GCF1* was expressed in *E. coli* as native or GST-fusion protein. (a) In contrast to uninduced cultures, crude protein extracts obtained from IPTG-induced cells contain a polypeptide (arrow) with a molecular mass corresponding to that of the GST-Gcf1 fusion protein. (b) Native (thrombin-cleaved) Gcf1p and GST-Gcf1p after purification on glutathione-agarose. (c) Gcf1 (40, 80, 120, 160, 200, 240, 280, 320 and 360 ng; lanes 2–10) protein was added to 300 ng *NdeI*-digested mtDNA of *C. albicans*. Lane 1, *NdeI*-digested mtDNA of *C. albicans* without addition of Gcf1p. Purified GST was used as a negative control (data not shown). (d) GST-Gcf1p (50 ng) was incubated with 40 ng terminally labelled fragments of mtDNA of *C. albicans* obtained by restriction with a combination of *Bam*HI and *Pvu*II in the absence or presence of nonlabelled mtDNA, as indicated. The numbers above the lanes indicate the molar excess of the competitor DNA. B, *Bam*HI; P, *Pvu*II.

### Recombinant Gcf1 protein binds DNA *in vitro*

To demonstrate that Gcf1 is a DNA-binding protein, the entire ORF was expressed in *E. coli* in fusion with N-terminally located GST (Fig. 3a). The crude protein fraction was loaded onto glutathione agarose, and Gcf1 was eluted either with glutathione (GST-Gcf1) or by thrombin digestion (native Gcf1) (Fig. 3b). To assess the DNA-binding properties of Gcf1p we employed an assay used by Sasaki *et al.* (2003), which demonstrates

preferential binding of Glom protein of *P. polycephalum* to certain regions of mtDNA. When we used purified mtDNA of *C. albicans* as a substrate, both GST-Gcf1 and native Gcf1 proteins exhibited DNA-binding activity *in vitro* when assayed by EMSA in agarose gels (Fig. 3c). GST alone at the same protein concentrations and under the same reaction conditions did not bind to DNA (data not shown). Using titrations of the Gcf1 protein in DNA-binding reactions we did not observe preferential binding

to any of the restriction fragments of *C. albicans* mtDNA. Similar results were obtained when we used digested plasmid DNA instead of mtDNA as a substrate (data not shown). Furthermore, we used radioactively labelled mtDNA fragments in the EMSA assay. When Gcf1p was incubated with digested and labelled mtDNA, most of the fragments dramatically reduced their mobility in agarose gels (Fig. 3d, lanes 1 and 2). Shorter fragments were shifted less efficiently, possibly indicating a cooperative binding of Gcf1p. Ten- and fivefold molar excess of unlabelled mtDNA fragments competed quantitatively with the labelled sample for complex formation (Fig. 3d, lanes 3 and 4)

There are two limitations of the interpretations of these *in vitro* data. First, Gcf1p expressed in *E. coli* may lack appropriate post-translational modifications, which may affect its biochemical properties in *C. albicans*. Second, the CUG codon in *C. albicans* encodes serine instead of leucine. In the case of *GCF1* there is a single CUG codon at position 21. However, substitution of serine for leucine at this position should not affect the DNA-binding properties of Gcf1p, since it lies within the N-terminal mitochondrial-targeting peptide and relatively far from the putative coiled-coil domain. In addition, note that the Ser<sup>21</sup>→Leu<sup>21</sup> substitution does not affect mitochondrial targeting in *S. cerevisiae* (Fig. 2a). Taken together, our results indicate that Gcf1p, similarly to Abf2p, is a non-specific DNA-binding protein potentially involved in packaging of *C. albicans* mtDNA.

### Complementation of *S. cerevisiae* $\Delta abf2$ mutant by *GCF1*

To analyse the functional similarity of the Gcf1 protein to *S. cerevisiae* Abf2p, we transformed wild-type as well as  $\Delta abf2$ -mutant *S. cerevisiae* strains with the expression plasmid pYES2/CT carrying *GCF1* under the control of the

*GAL1* promoter. We took advantage of the fact that whereas cells lacking Abf2p rapidly lose mtDNA when grown on fermentable carbon sources (e.g. glucose or galactose), they remain respiratory-competent on a non-fermentable carbon source (e.g., glycerol) due to the activity of Aco1 protein (Chen *et al.*, 2005). We observed that expression of *GCF1* almost completely prevented loss of mtDNA when the transformants were grown on synthetic media containing galactose (Table 2). Similar results were obtained on media containing glycerol, which is consistent with the derepression of the *GAL1* promoter under these conditions. In contrast, propagation of the transformants on glucose media resulted in a massive loss of mtDNA. These results indicate that Gcf1p function is able to partially substitute the function of the Abf2 protein in *S. cerevisiae*. Interestingly, galactose-induced expression of Gcf1 in a *S. cerevisiae* strain containing a wild-type *ABF2* allele substantially increased the frequency of petite colonies (Table 2). Similar results were obtained when we performed the complementation experiment with *GCF1* from *C. parapsilosis* (Supplementary Table S1). This suggests that co-expression of *ABF2* and *GCF1* leads to competition of these proteins for mtDNA substrate and a subsequent destabilization of the mitochondrial genome.

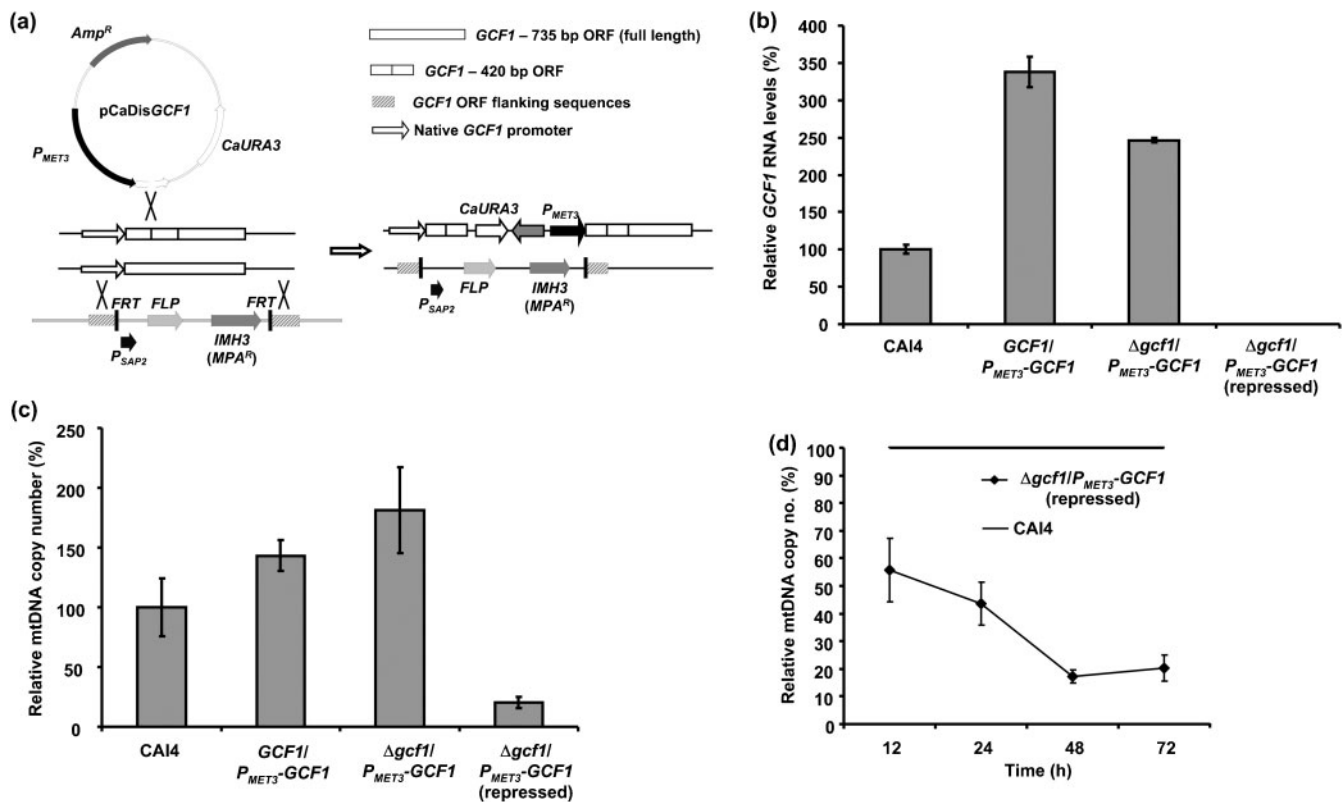
### Construction of a *C. albicans* strain with a conditional allele of *GCF1* and its phenotypic analysis

To analyse the role of *GCF1* in mtDNA metabolism in *C. albicans*, we constructed a strain which lacks one allele of *GCF1* and in which the second allele is replaced by a locus enabling conditional expression of *GCF1* from the *MET3* promoter repressible by 2.5 mM methionine/cysteine in the medium (Care *et al.*, 1999; Fig. 4a). The correct insertion of both constructs into the genome was verified by both PCR and Southern blot analyses (data not shown).

**Table 2.** Complementation of *S. cerevisiae*  $\Delta abf2$  mutant by *GCF1*

Strain	<i>ABF2</i> allele	Plasmid	Medium*	Number of colonies (%)	
				Grande	Petite
YAM 101-1a	<i>ABF2</i>	pYES2/CT	SD	99.7 ± 0.3	0.3 ± 0.3
YAM 101-1a	<i>ABF2</i>	pYES2/CT	SG	98.2 ± 1.6	1.8 ± 1.6
YAM 101-1a	<i>ABF2</i>	pYES2/CT	SGal	94.0 ± 6.1	6.1 ± 6.1
YAM 101-1a	<i>ABF2</i>	pYES2/CT- <i>GCF1</i>	SD	99.2 ± 0.9	0.9 ± 0.9
YAM 101-1a	<i>ABF2</i>	pYES2/CT- <i>GCF1</i>	SG	99.4 ± 0.6	0.6 ± 0.6
YAM 101-1a	<i>ABF2</i>	pYES2/CT- <i>GCF1</i>	SGal	48.3 ± 2.4	51.7 ± 2.4
YAM 101-1b	$\Delta abf2$	pYES2/CT	SD	2.7 ± 0.9	97.3 ± 0.9
YAM 101-1b	$\Delta abf2$	pYES2/CT	SG	22.2 ± 20.7	77.8 ± 20.7
YAM 101-1b	$\Delta abf2$	pYES2/CT	SGal	9.0 ± 4.5	91.0 ± 4.5
YAM 101-1b	$\Delta abf2$	pYES2/CT- <i>GCF1</i>	SD	12.2 ± 3.3	87.8 ± 3.3
YAM 101-1b	$\Delta abf2$	pYES2/CT- <i>GCF1</i>	SG	77.2 ± 6.9	22.8 ± 6.9
YAM 101-1b	$\Delta abf2$	pYES2/CT- <i>GCF1</i>	SGal	52.6 ± 17.6	47.4 ± 17.6

\*Synthetic medium containing glucose (SD), glycerol (SG) or galactose (SGal) as carbon source.



**Fig. 4.** Schematic illustration of preparation of the strain carrying one conditional allele of *GCF1*, quantification of mRNA levels and mtDNA copy numbers. (a) One allele of *GCF1* was disrupted by integration of plasmid pCaDisGCF1 into the *GCF1* ORF by homologous recombination of a short coding region (400 bp). The second allele was replaced by the *MPA<sup>R</sup>-FLP* cassette containing sequences homologous to flanking sequences of the *GCF1* ORF at both sides of the cassette. The arrow is pointing at a schematic structure of a double mutant of *GCF1* lacking one allele and containing the *MET3*-driven second allele of *GCF1*. FRT, FLP recombination target; FLP, flip-flop recombinase; P, promoter. (b) Relative *GCF1* mRNA levels of strains CAI4, *GCF1/P<sub>MET3</sub>-GCF1* and  $\Delta$ *gcf1/P<sub>MET3</sub>-GCF1* grown under derepressed and repressed conditions as determined by qRT-PCR, normalized using *CaACT1* as a standard. *GCF1/P<sub>MET3</sub>-GCF1* and  $\Delta$ *gcf1/P<sub>MET3</sub>-GCF1* grown under derepressed conditions show two- to threefold increases in *GCF1* mRNA compared with the wild-type, while repression of the *MET3* promoter-controlled *GCF1* allele resulted in an approximately 3200-fold decrease in *GCF1* mRNA compared with the wild-type. Error bars, SD. (c) Relative mtDNA copy numbers of strains CAI4, *GCF1/P<sub>MET3</sub>-GCF1* and  $\Delta$ *gcf1/P<sub>MET3</sub>-GCF1* grown under derepressed and repressed conditions as determined by qRT-PCR; normalization was carried out using *CaACT1* as a standard. Error bars, SD. In strains *GCF1/P<sub>MET3</sub>-GCF1* and  $\Delta$ *gcf1/P<sub>MET3</sub>-GCF1* grown under derepressed conditions, average mtDNA copy numbers are elevated by 25–50%, whereas the strain  $\Delta$ *gcf1/P<sub>MET3</sub>-GCF1* grown under repressed conditions for 48 h exhibits an approximately 80% reduction in the number of mtDNA copies compared with the wild-type. (d) Relative mtDNA copy numbers of strain  $\Delta$ *gcf1/P<sub>MET3</sub>-GCF1* grown under repressed conditions, and passaged every 12 h for 3 days, ensuring linear growth to OD<sub>600</sub> 1.0 at each sampling time point. Twelve hours of growth under repressed conditions already decreases mtDNA copy numbers by 40%, and further passaging reveals a reduction to 20% of wild-type level after 48 h. Error bars, SD. In (b), (c) and (d), results for the CAI4 wild-type strain were defined as 100%.

The resulting strain  $\Delta$ *gcf1/P<sub>MET3</sub>-GCF1* was able to grow under both repressed and derepressed conditions. *GCF1* mRNA levels in the wild-type (CAI4), *GCF1/P<sub>MET3</sub>-GCF1* and  $\Delta$ *gcf1/P<sub>MET3</sub>-GCF1* grown under repressed and derepressed conditions were analysed by qRT-PCR. *CaACT1* mRNA encoding actin was used as a standard for normalization. *GCF1* mRNA levels in the repressed strain  $\Delta$ *gcf1/P<sub>MET3</sub>-GCF1* were 3200-fold ( $\pm 14\%$ ) lower than in the wild-type strain after 12 h of growth in repression medium containing Met/Cys (Fig. 4b). Interestingly, both

*GCF1/P<sub>MET3</sub>-GCF1* and  $\Delta$ *gcf1/P<sub>MET3</sub>-GCF1* strains grown under derepressed conditions exhibited two- to threefold elevated levels of *GCF1* mRNA compared with the wild-type strain (Fig. 4b). Relative copy numbers of mtDNA in strains CAI4, *GCF1/P<sub>MET3</sub>-GCF1* and  $\Delta$ *gcf1/P<sub>MET3</sub>-GCF1* were determined by qRT-PCR. As indicated in Fig. 4(c), the copy number of mtDNA in the repressed strain  $\Delta$ *gcf1/P<sub>MET3</sub>-GCF1* was reduced by 80% compared with the wild-type strain, whereas both the *GCF1/P<sub>MET3</sub>-GCF1* and the  $\Delta$ *gcf1/P<sub>MET3</sub>-GCF1* strains grown under derepressed

conditions contained up to 50% more mtDNA. Clearly, the elevated levels of *GCF1*-mRNA in the cells correlate with the observed higher copy numbers of mtDNA. Similarly, it has been demonstrated that moderate over-expression of *S. cerevisiae* *ABF2* increases the amount of mtDNA by 50–100% (Zelenaya-Troitskaya *et al.*, 1998). On the other hand, *S. cerevisiae* cells lacking *ABF2* exhibit rapid loss of mtDNA on fermentable carbon sources (e.g. glucose). Only when grown on non-fermentable carbon sources (e.g. glycerol) can  $\Delta abf2$  strains of *S. cerevisiae* maintain their mtDNA (Diffley & Stillman, 1991). *C. albicans* is a petite-negative yeast, which cannot tolerate alteration or loss of its mtDNA. We were unable to knockout both alleles of *GCF1*. Although this could be due to technical reasons, it is possible and more likely that a strain lacking both functional alleles of *GCF1* is not viable. Moreover, growing  $\Delta gcfl/P_{MET3}$ -*GCF1* under repressed conditions in synthetic minimal medium affected growth and slowed the cell cycle down three- to fivefold. Considering these observations and our data on *GCF1* mRNA levels and mtDNA copy numbers it is plausible to propose that (i) *GCF1* is an essential gene for mtDNA maintenance in *C. albicans*, and (ii) very low levels of *GCF1* mRNA (and thus Gcf1 protein) could be sufficient to maintain levels of mtDNA that keep *GCF1*-shut-off mutants viable. This hypothesis is supported by our data shown in Fig. 4(d). Strain  $\Delta gcfl/P_{MET3}$ -*GCF1*, grown under repressed conditions, was passaged every 12 h for 3 days keeping the cultures in linear growth phase up to OD<sub>600</sub> 1.0. Analyses of samples taken at different time points show that during the first 24 h of passaging the cells, mtDNA copy numbers, as analysed by qRT-PCR, in this strain dropped down to 40% of wild-type level. During the following 24 h, copy numbers dropped further, reaching a plateau at 20% of wild-type level. This can be interpreted as follows: low *GCF1* mRNA levels ensure a critical minimum level of Gcf1 protein, which in return supports maintenance of a vital amount of mtDNA in *C. albicans*. However, we cannot rule out the possibility that the vitality of the  $\Delta gcfl/P_{MET3}$ -*GCF1* strain under repressed conditions is caused by alternative protein factors (e.g. Aco1p) that could stabilize mtDNA in the absence of *GCF1*, as has been described for *S. cerevisiae* (Chen *et al.*, 2005). In any case, a regulated expression of *GCF1* enabled us to study the consequences of decreased levels of this protein on metabolism of mtDNA in *C. albicans*.

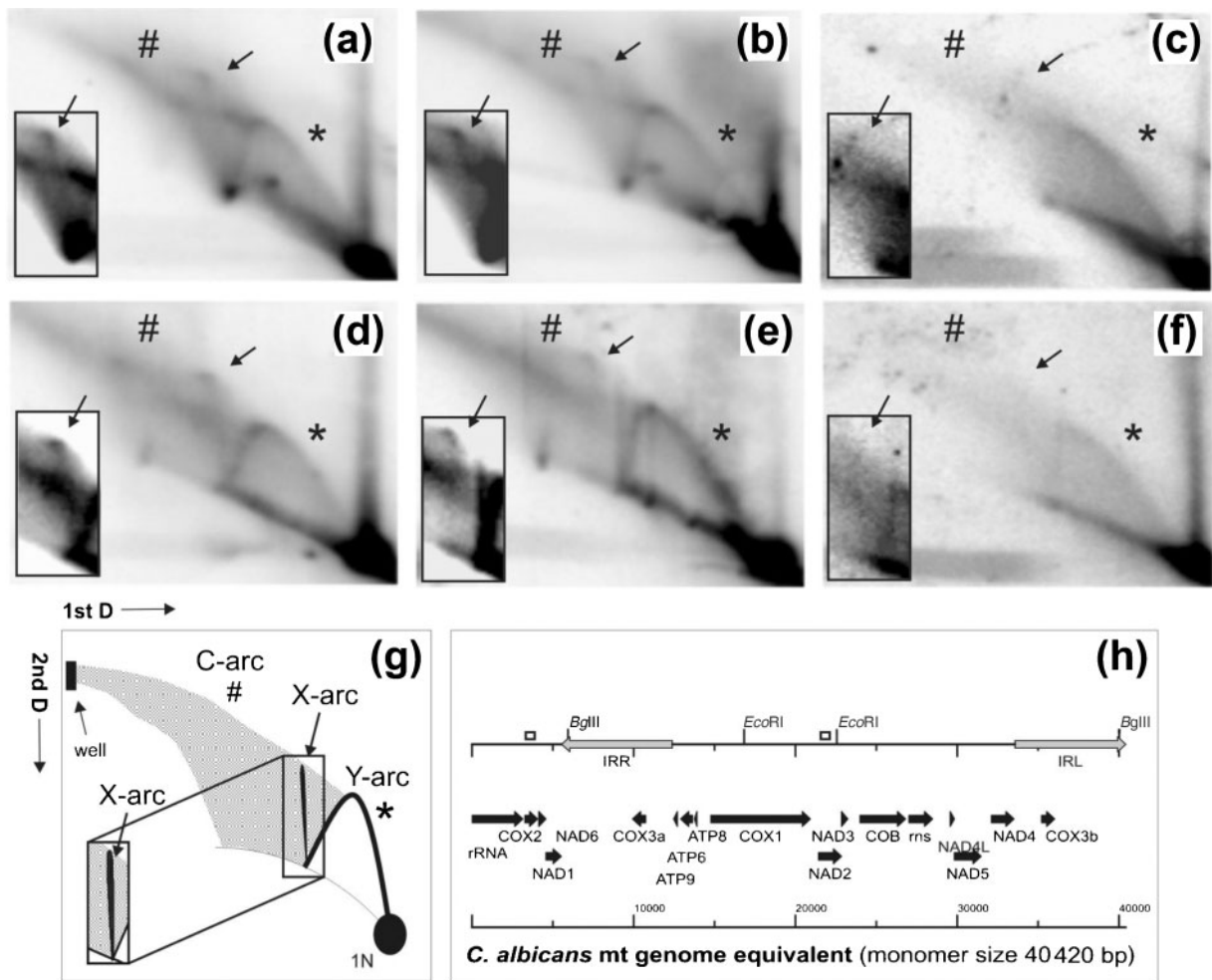
In order to demonstrate a direct effect of *GCF1* on mtDNA metabolism in *C. albicans* we used 2D-AGE to analyse metabolic intermediates of mtDNA. In *S. cerevisiae*, the Abf2 protein binds directly to mtDNA, leading to its bending and compaction, and it has been indicated to play a role in mtDNA recombination (Stigter, 2004; Friddle *et al.*, 2004; Zelenaya-Troitskaya *et al.*, 1998). 2D-AGE is a powerful tool to investigate the topology of DNA molecules by separating them in the first dimension according to their mass under low voltage and in the absence of intercalating chemicals, and in the second

dimension under higher voltage and using ethidium bromide to separate molecules according to their mass and shape. If DNA to be analysed is digested with restriction endonucleases prior to 2D-AGE, replication and recombination intermediates can be discriminated on the basis of a standard Y-arc formed by replication forks passing the restriction fragment analysed, an X-arc formed by Holliday junctions within the fragment, and an arc extending from larger linear double-stranded molecules to the well (C-arc), believed to be made up of highly branched complex recombination intermediates (Manchekar *et al.*, 2006; J. M. Gerhold and others, unpublished results). 2D-AGE analyses of mtDNA from strain  $\Delta gcfl/P_{MET3}$ -*GCF1* grown under repressed conditions revealed a significant decrease in high-molecular-mass recombination structures and the absence of Holliday junctions (Fig. 5c, f) compared to the wild-type and  $\Delta gcfl/P_{MET3}$ -*GCF1* grown under derepressed conditions (Fig. 5a, b, d, e). The severe reduction of Gcf1p levels in *C. albicans* mitochondria under repressed conditions apparently leads to the observed decrease in recombination structures that could be used for initiation of mtDNA replication. Recently, it has been demonstrated that in yeast recombination is linked to the initiation of mtDNA replication (Ling *et al.*, 2007; J. M. Gerhold and others, unpublished results). While the mechanistic details of this process are as yet unclear, it is most likely to be similar to the recombination-based replication observed in bacteriophage T4 (Mosig, 1998; Kreuzer *et al.*, 1995). It is possible that the Gcf1 protein facilitates recombination by bending mtDNA and bringing together homologous DNA regions and proteins required for initiation of the process. Due to the reduced frequency of mtDNA recombination events in the repressed strain  $\Delta gcfl/P_{MET3}$ -*GCF1*, the rate of mtDNA synthesis drops and the accumulation of vital levels of mtDNA takes longer than usually assumed for the wild-type cells. Therefore, cell cycle progression is slowed down.

As an alternative to a role in mtDNA replication initiation, a possible compaction function of Gcf1p together with its DNA-bending properties might influence mtDNA segregation and thereby slow down the cell cycle in the repressed  $\Delta gcfl/P_{MET3}$ -*GCF1* strain. However, DAPI staining of the repressed  $\Delta gcfl/P_{MET3}$ -*GCF1* strain did not indicate substantial changes in mt-nucleoid morphology (data not shown), and suggests therefore that the Gcf1 protein is dispensable for segregation of mtDNA in *C. albicans*.

## Conclusions

We have characterized Gcf1p of *C. albicans*, a member of a novel subfamily of mtHMGs. Our results indicate that similarly to *S. cerevisiae* Abf2p, Gcf1p is a non-specific DNA-binding protein involved in mtDNA replication and recombination, and thus in the fine-tuning of mtDNA metabolism. It remains to be determined if and how other components of *C. albicans* mt-nucleoid participate in its function(s). One obvious candidate could be aconitase,



**Fig. 5.** 2D electrophoresis. Purified mtDNA of CAI4 (a, d),  $\Delta gcf1/PMET3-GCF1$  grown under derepressed conditions (b, e) and  $\Delta gcf1/PMET3-GCF1$  grown under repressed conditions (c, f) was digested with *Bgl*II (a, b, c; fragment size 6328 bp, spanning base pairs 40 026 to 5934 *in vivo*) probed for a portion of *cox2* (probe spanning base pairs 3285 to 3654), and *Eco*RI (d, e, f; fragment size 5726 bp, spanning base pairs 16 828 to 22 554) probed for *nad2* (probe spanning base pairs 21 500 to 21 850). Positions of probes for *cox2* and *nad2* are indicated as open boxes in (h). (g) Graphical interpretation of (a–f). (h) An overview of a one-genome equivalent (monomer; 40 420 bp) of the *C. albicans* mitochondrial genome. Radiographs reveal a standard Y-arc [indicated by an asterisk; interpreted in (g)] of strand-coupled replication intermediates on all panels, albeit at reduced strength in (c) and (f). Each panel contains an overexposed section displaying the region of the descending Y-arc to the 2N spot (an almost fully replicated restriction fragment) and X-arc (indicated by an arrow) of Holliday junctions. In strain  $\Delta gcf1/PMET3-GCF1$  grown under repressed conditions (c, f), X-arcs are reduced to a level below detection, and the C-arcs (indicated by #), which consist of complex high-molecular-mass recombination structures and branched molecules, are significantly reduced in comparison to CAI4 (a, d) and  $\Delta gcf1/PMET3-GCF1$  grown under derepressed conditions (b, e).

which substitutes for a lack of Abf2p on non-fermentable carbon sources in *S. cerevisiae* (Chen *et al.*, 2005). Indeed, Aco1p has been detected in preparations of mt-nucleoids in *C. parapsilosis* (Miyakawa *et al.*, 2009). In any case, our results clearly demonstrate the importance of Gcf1p for optimal growth yields, and the data on mtDNA copy number, such as topological studies of replication and recombination intermediates, underline the effect of this protein on mtDNA metabolism.

Our paper underlines the relatively high rates of divergence of yeast mtHMGs (Nosek *et al.*, 2006). The degree of sequence variability between the members of the four subfamilies of mtHMGs is so high that it is difficult to reconstruct their evolutionary history. One possibility is that the HMG boxes are shuffled between the proteins involved in mtDNA packaging. According to another scenario, the class IIb mtHMGs, containing two HMG boxes and one coiled-coil domain, may represent an

ancestral type of mtHMG. Comparative analysis of Gcf1p with other members of the novel family of mtHMGs (*CpGcf1*, *DhGcf1*) as well as with more distantly related mtDNA packaging proteins, including Abf2, may lead to a better understanding of the means of stabilization and transmission of mtDNA in yeasts.

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