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Research Article

A YEAST SPECIFIC INSERTION AMIDST OBG FOLD IS CRITICAL FOR THE MITOCHONDRIAL FUNCTION OF Mtg2p IN *SACCHAROMYCES CEREVISIAE*

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Abstract: Protein expression in mitochondria is carried out by ribosomes that are distinct from their cytosolic counterpart. Mitochondrial ribosomes are made of individual proteins having distinct lineages: those with clear bacterial orthologues, those conserved in eukaryotes only and proteins that are species specific. *MTG2* is the mitochondrial member of the universally conserved Obg family of GTPases in *Saccharomyces cerevisiae* which associates with and regulates mitochondrial large ribosomal subunit assembly. In this study we demonstrate that *MTG2*, in addition to the universally conserved OBG and GTPase domains, has an essential yeast specific insertion domain positioned within the N terminal OBG fold. Cells expressing $mtg2\Delta_{201\text{-}294}$, deleted for the insertion domain are not able to support cellular respiration. In addition, we show that large stretches of amino acids can be inserted into *MTG2* at the end of the yeast specific insertion domain and the OBG fold without perturbing its cellular functions, consistent with the insertion domain folding into a species specific protein binding platform.

Keywords: mitochondrial ribosome; Mtg2p/Obg

Note : *Coloured Figures available on Journal Website in "Archives" Section*

Introduction

Mitochondria are central to metabolism, catabolism, organellar and intracellular communication besides being the powerhouse of the cell where majority of ATP is synthesized. Mitochondria require coordinated gene expression from two spatially distinct genetic material, nuclear DNA and mitochondrial (mt) DNA. In *Saccharomyces cerevisiae*, mtDNA encodes for eight polypeptides, all being a part of the electron transport chain with the exception of *VAR1* (Kurland and Andersson, 2000). For gene expression mitochondria maintains its own distinct ribosomes composed of a small 37S subunit and a large 54S subunit which comes together during translation to form a 74S monosome. While 15S and 21S rRNAs, which are components of small and large subunit respectively,*VAR1,* an essential protein of the small ribosomal subunit are encoded by the mtDNA (Foury *et al.*, 1998), approximately 90 mitochondrial ribosomal proteins (MRP) and assembly factors are encoded by a set of nuclear genes, separate from those encoding for cytosolic ribosomes (Graack and Wittmann-Liebold, 1998). Assembly of mitochondrial ribosomes requires the coordinated synthesis of mitochondrially encoded rRNAs and nuclear encoded proteins, the import of these cytosolic proteins into the mitochondria and their association into functional ribosomal subunits.

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Mitochondrial ribosomes are structurally distinct from their bacterial and cytosolic counterparts with a high degree of variability among different species (Amunts *et al.*, 2014; Perocchi *et al.*, 2008). During mitochondrial evolution, the RNA content of the ribosome has reduced from 65% in bacteria to approximately 31% in mammalian mitochondria (Amunts *et al.*, 2014; Mears *et al.*, 2006; Sharma *et al.*, 2003). To compensate for reduction in the rRNA size, there is an increase in the protein mass, thereby increasing protein to RNA mass ratio (Amunts *et al.*, 2014; Cavdar Koc *et al.*, 2001; Koc *et al.*, 2001; O'Brien, 2002). These mitochondrial specific ribosomal proteins, in addition to their role in maintaining the structural features of the core ribosome, are also required for mitochondrial mRNA recognition, association with the mitochondrial inner membrane and for nascent chain insertion into the inner membrane, resulting in functional diversity observed in the mitochondrial translation system(Bonnefoy *et al.*, 2009; Fox, 1996; Green-Willms *et al.*, 1998; Ott and Herrmann, 2010; Williams *et al.*, 2005; Williams *et al.*, 2004).

Ribosome biogenesis is a complex multistep process involving temporal processing and modification of rRNA as well as coordinated loading of ribosomal proteins onto rRNA to form functional small and large (37S and 54S in case of mitochondria) subunits. This requires numerous auxiliary factors that aid in maturation of precursor molecules into functional subunits without being a part of the core structure. These assembly factors include nucleases, RNA helicases and modifying enzymes, as well as monomeric GTPases (De Silva *et al.*, 2015; Kressler *et al.*, 2010; Strunk and Karbstein, 2009). All organisms possess a number of conserved monomeric GTPases that are predicted to have evolved from an ancestral GTPase involved in translation including *MTG2* (Leipe *et al.*, 2002).

MTG2 is the mitochondrial member of the Obg family of GTPase. Mtg2p is essential for mitochondrial function. It has been shown to co-purify with pre54S and regulate a late step of mitochondrial large subunit assembly in *S. cerevisiae* (Datta *et al.*, 2005). Obg family of GTPases been found in all organisms sequenced to date and all Obg proteins studied till date are involved in some aspect of ribosome function. Prokaryotes have one Obg protein, whereas eukaryotes have four paralogs (Leipe *et al.*, 2002). All Obg proteins possess a highly conserved GTP-binding domain, suggesting that they share a common mode of regulation. In *S. cerevisiae*, each distinct Obg protein type appears to play a specialized role in ribosome function. The nucleolar Nog1p protein co-purifies with pre-60S intermediates and is involved in cytosolic ribosome assembly, particularly in biogenesis of the pre 60S particle (Fromont-Racine *et al.*, 2003; Kallstrom *et al.*, 2003; Saveanu *et al.*, 2003). In contrast, Rbg1p and Rbg2p, two cytosolic Obg proteins that are 52% similar, associate with translating ribosomes and linked to sensing stress response (Wout *et al.*, 2009).

Mitochondrial and bacterial Obg proteins are likely to be orthologous as they are conserved throughout their protein lengths. In contrast, the other three eukaryotic Obg proteins possess vastly different protein sequences outside of the GTP binding domain (Leipe *et al.*, 2002). Mtg2p has similar domain organization as the bacterial Obg protein i.e. an N-terminal glycine rich OBG domain followed by a GTPase domain (Buglino *et al.*, 2002; Gkekas *et al.*, 2017; Kukimoto-Niino *et al.*, 2004). Mtg2p in yeast has an additional insertion within the OBG fold besides an N-terminal mitochondrial targeting sequence. Similar insertions are largely conserved in mitochondrial Obg members from other fungal species but absent in mitochondrial Obg members of other eukaryotes. In this study we show that the yeast insertion domain is essential for *MTG2* function *in vivo*. We also show that large amino acid sequences can be inserted at the end of this domain without disrupting *MTG2* function.

Materials and Methods

Yeast strains and media

Complete media used were 1% yeast extract, 2% peptone containing 2% glucose (YPD) or 3% glycerol (YPG) as carbon source. Synthetic minimal media (0.67% yeast nitrogen base without amino acids containing 2% glucose [SD], 0.1% 5 fluoro orotic acid, 2% glucose [5FOAD]) were supplemented with appropriate amino acids, as described (Guthrie and Fink, 1991).

Plasmid and strain construction

All manipulations regarding genomic DNA isolation and transformation of yeast with plasmid DNA were carried out as described (Guthrie and Fink, 1991). Episomal plasmids expressing *MTG2*

under its endogenous promoter, pKD5 (*pRS316::MTG2*) and pKD7 (*pRS313::MTG2*) and JM2195, a ρ^+ strain with *mtg2::kanMX4* allele on the chromosome, harboring pKD5were obtained from (Datta *et al.*, 2005).

Plasmids expressing *MTG2* tagged with Tandem affinity purification (TAP)tag or Green Fluorescent Protein (GFP) between amino acid residues 294-295 under its endogenous promoter were created as follows:

pRS313::MTG2*$_{294\text{-}295}$TAP:TAP* tag was amplified from pBS1479 (Rigaut *et al.*, 1999) using polymerase chain reaction (PCR) with oligonucleotides

"5'TGGAGGATCCATGGAAAAGAGAAGATG3'" and

"5'TCAGGTAACGGTTGACTTCCCCGCGGAA3'".

The 566bp PCR product was cloned into LITMUS29 as a BamHI-KpnI fragment to yield pKD32. The N-terminal of *MTG2* gene encoding residues 1-294, was amplified from pKD5 using PCR with oligonucleotides

"5'GGCACGTGCCAAATTTG3'" and

5'CGGGATCCTGGTTTCGTCATTGGCTG3'.

The 1377bp PCR product was cloned into pCR®2.1 TOPO (Invitrogen) to yield pKD25. The *mtg2*$_{1\text{-}294}$ was sub-cloned into pKD32 as an EcoRV-BamHI to yield pKD34. The C-terminal of *MTG2* gene encoding residues 294-518, was amplified from pKD5 using PCR with oligonucleotides

"5'CGGGGTACCGTATGTCTGCTGAAAGGTGG3'" and "5'GCATAGGCCCTGCAAAT3'". The 1112bp PCR product was cloned into pCR®2.1 TOPO (Invitrogen) to yield pKD30. The *mtg2*$_{294\text{-}518}$ was sub-cloned into pKD34 as a KpnI-SpeI to yield pKD35. *MTG2*, with TAP inserted in the correct reading frame between residues 294-295 was sub-cloned from pKD35 into pKD7 (*pRS313::MTG2*) as a SphI-AatII to yield pKD36.

pRS313::MTG2*$_{294\text{-}295}$GFP:GFP* was amplified from pFA6a-*GFP(S65T)*-His3MX6 (Longtine *et al.*, 1998) using PCR with oligonucleotides

"5'CAGGTCGACGGATCCCCGGG3'" and

"5'TCAGGTAACTTTGTGTAGTTCATCCAT3'".

The 781bp PCR product was cloned into LITMUS29 as a BamHI-KpnI fragment to yield pKD37. The N-terminal of *MTG2* gene encoding residues 1-294, was amplified from pKD5 using PCR with oligonucleotides

"5'GGCACGTGCCAAATTTG3'" and

"5'CGGGATCCGTGGTTTCGTCATTGGCTG3'".

The 1377bp PCR product was cloned into pCR®2.1 TOPO (Invitrogen)to yield pKD27. The *mtg2*$_{1\text{-}294}$ was sub-cloned into pKD37 as an EcoRV-BamHI to yield pKD39. The C-terminal of *MTG2* gene encoding residues 294-518, was sub-cloned into pKD39 as a KpnI-SpeI to yield pKD40. *MTG2*, with *GFP* inserted in the correct reading frame between residues 294-295 was sub-cloned from pKD40 into pKD7 (*pRS313::MTG2*) as a SphI-AatII to yield pKD41.

***pRS313::mtg2*$\Delta_{201\text{-}294}$:** Plasmid expressing *mtg2* deleted for amino acid residues 201-294 under its endogenous promoter were created as follows: The C-terminal of *MTG2* gene encoding residues 295-518, was amplified from pKD5 using PCR with oligonucleotides

"5'CATATGGTATGTCTGCTGAAAGGTGG3'" and

"5'GCATAGGCCCTGCAAAT3'". The 1102bp PCR product was cloned into pCR®2.1 TOPO (Invitrogen) to yield pKD16. The N-terminal of *MTG2* gene encoding residues 1-294, was amplified from pKD5 using PCR with oligonucleotides

"5'GGCACGTGCCAAATTTG3'" and

"5'GGCATTTGTTTGCGCATTTCTCTTTCCA3'".

The1100bp PCR product was cloned into pCR®2.1 TOPO (Invitrogen) to yield pKD28. The *MTG*$_{295\text{-}518}$ was sub-cloned from into pKD28 into pKD16 as NdeI-XhoI fragment to yield pKD46. The *mtg2*$\Delta_{201\text{-}294}$was constructed by sub-cloning a 384bp region encompassing the deleted nucleotide from pKD46 into pKD7 as a SphI-AatII fragment to create pKD47.

Complementation assay

In order to determine whether various alleles of *MTG2* created are functional, pKD36, pKD41 and pKD47 were transformed into JM2195. Transformants were selected on SD medium lacking uracil and histidine. Transformants were patched onto SD medium containing 0.1% 5 fluoro orotic acid, to counter-select for pKD5. Cells were then grown in YPD medium prior to testing for their growth on YPG medium.

Structure prediction

In order to predict the structure of Mtg2p and $CgtA_C$, sequence was analysed using Phyre2 web portal (Kelley *et al.*, 2015) at *http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index*. UCSF Chimera (Pettersen *et al.*, 2004) *http://www.rbvi.ucsf.edu/chimera* was used to render different domains in their respective colour.

Results and Discussion

In *Saccharomyces cerevisiae*, *MTG2* is the mitochondrial member of the Obg family of GTPases which are universally conserved throughout evolution containing an N-terminal glycine rich OBG domain followed by a GTPases domain. Comparison of Mtg2p from *Saccharomyces cerevisiae* with Obg protein sequences from *Escherichia coli*, *Caulobacter crescentus*, *Homo sapiens*, *Drosophila melanogaster* indicate some novel features for Mtg2p. First, Mtg2p possesses 88 N terminal amino acids not found in the bacterial proteins, which is predicted to contain a cleavable mitochondrial targeting sequence (https://ihg.gsf.de/ihg/mitoprot.html) (Claros and Vincens, 1996). Similar cleavable mitochondrial sequences are predicted in other eukaryotic Obg proteins as well. Mtg2p terminates shortly after the conserved GTP-binding domain, whereas many Obg proteins of the prokaryotic lineage have additional C-terminal sequences. Interestingly, the C-terminal domains of bacterial Obg proteins are highly diverse and thought to interact with stress response pathways specific for individual species (Feng *et al.*, 2014; Scott *et al.*, 2000). Second, Mtg2p includes a short insertion between amino acids 201 and 294 positioned between conserved structural elements within the extended OBG domain (Figure 1). Similar insertions are found in mitochondrial members of Obg proteins in fungi but absent in mitochondrial Obg members of other eukaryotes (Figure 1).Crystal structure of bacterial Obg proteins from *Escherichia coli*, *Bacillus subtilis* and *Thermus thermophilus* indicates three features within the OBG domain: six glycine rich left handed type II helices at the centre, loops connecting the type II helices on one end and eight β sheets and one α helix on the other (Buglino *et al.*, 2002; Gkekas *et al.*, 2017; Kukimoto-Niino *et al.*, 2004). Comparison of Phyre2 predicted structure of Mtg2p and $CgtA_C$ indicates that Mtg2p has overall similar domain organization as the bacterial Obg proteins with the G2 sequence in close proximity to OBG fold (Figure 2). The yeast specific insertion can be placed in between the seventh β sheet and fifth type II helix within the OBG fold (Figure 2).

In order to test whether the yeast specific insertion is essential for *in vivo MTG2* function, we tested the ability of an allele of *MTG2* deleted for amino acid residues 201-294 to complement a Δ*mtg2* ρ^+ strain. Cells harbouring $mtg2\Delta_{201\text{-}294}$ allele were not able to utilize glycerol as the sole carbon source in comparison to *MTG2*, indicating that this insertions is essential for Mtg2p function (Figure 3). Interestingly all yeast species examined so far have an insertion domain within the OBG fold (Figure 4). Although they vary in size and sequence conservation, a unifying feature is a conserved proline residue (residue 294 in Mtg2p) at the junction of the insertion domain and the fifth type II helix of the Obg fold (Figure 4). Given that a branch of life is able to tolerate insertions within the Obg fold, we tested whether additional sequences could be inserted at this junction in *MTG2*. Sequences expressing either Tandem affinity purification (TAP) tag or Green fluorescent protein(GFP) were inserted in frame between proline^{294} and valine^{295} of Mtg2p. Stop codon of TAP and GFP was replaced with a glycine residue. To test whether $MTG2{:}TAP_{294\text{-}295}$ and $MTG2{:}GFP_{294\text{-}295}$ were functional *in vivo*, ability to utilize glycerol as the sole carbon source were tested in cells expressing them as the sole copy of *MTG2*. Cells harbouring $MTG2{:}TAP_{294\text{-}295}$ and $MTG2{:}GFP_{294\text{-}295}$ alleles as the sole copy in a Δ*mtg2* ρ^+ strain were able to utilize glycerol as the sole carbon source at the same rate as cells harbouring wild type allele of *MTG2* (Figure 5), indicating that relatively large protein insertions at the end of this yeast specific insertion region did not perturb Mtg2p function.

Mitochondrial ribosomes are made up of proteins that have clear bacterial homologues. A handful of mitochondrial ribosomal proteins on the other hand have evolved additional domains fused to the conserved bacterial protein (Amunts *et al.*, 2014; Kaur and Stuart, 2011; Williams *et al.*, 2004). These proteins, in addition to the conserved function in translation also perform specialized functions through these inserted domains that add to diversity of the mitochondrial translation system. For example, MrpL36p contains a C-terminal extension (CE) domain in addition to the conserved L31 domain found in bacterial ribosomal large-subunit protein. The L31 region of MrpL36p is

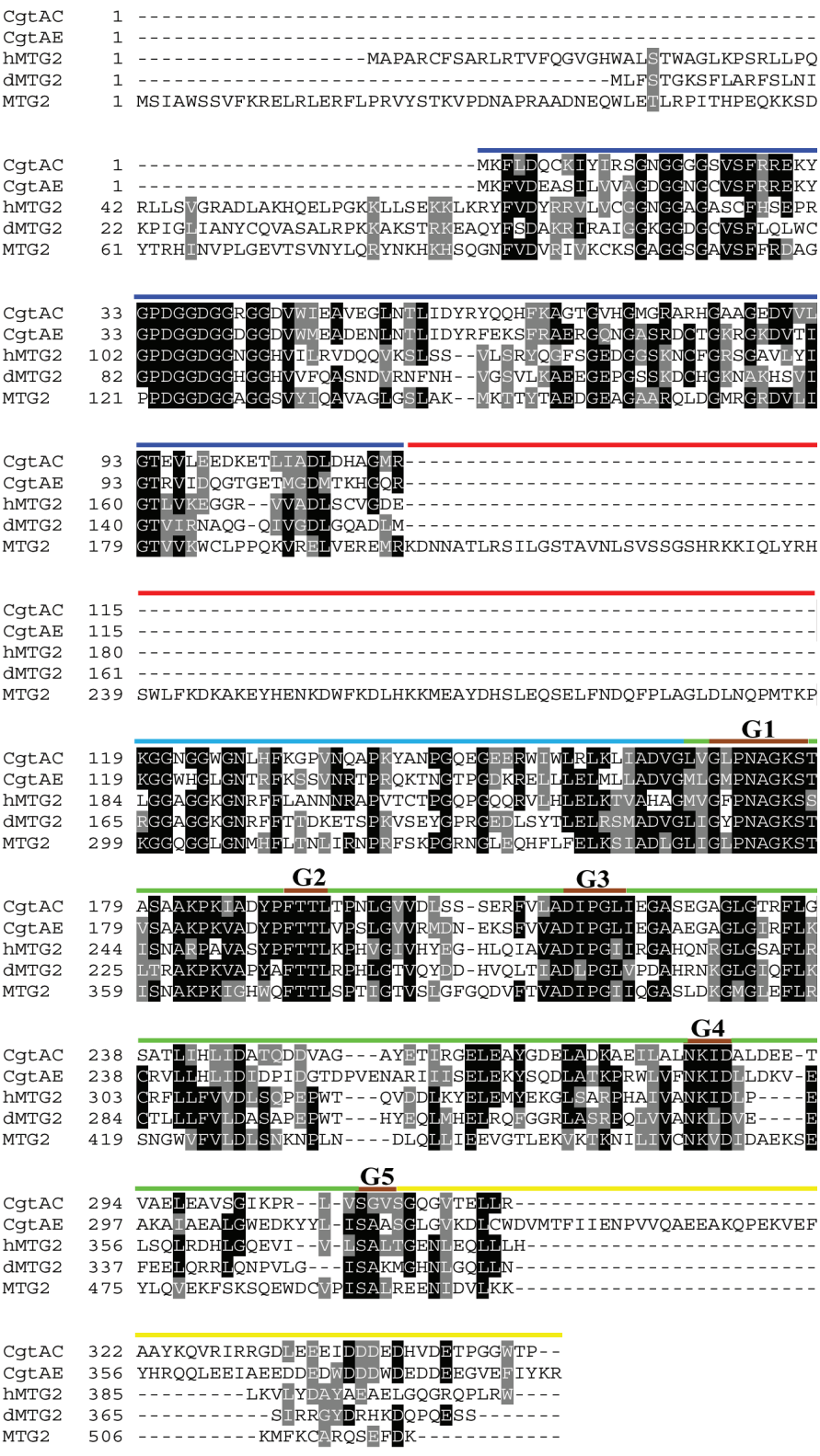

Figure 1: **Comparison of Obg proteins. Identical amino acids are boxed in black whereas conserved amino acids are shaded. Sequences include Obg proteins from *Caulobater crescentus* (CgtA$_C$), *Escherichia coli* (CgtA$_E$), *Homo sapiens* (hMTG2), *Drosophilla melanogaster* (dMTG2) and *Saccharomyces cerevisiae* (MTG2). The GTP binding domain is marked in green with motifs (G1-G5) (Bourne *et al.*, 1991) marked with brown lines. The OBG fold (Buglino *et al.*, 2002) before and after the insertion sequence are indicated with a blue and turquoise line, respectively. The insertion region in *MTG2* is indicated with a red line. The C-terminal region is indicated with a yellow line.**

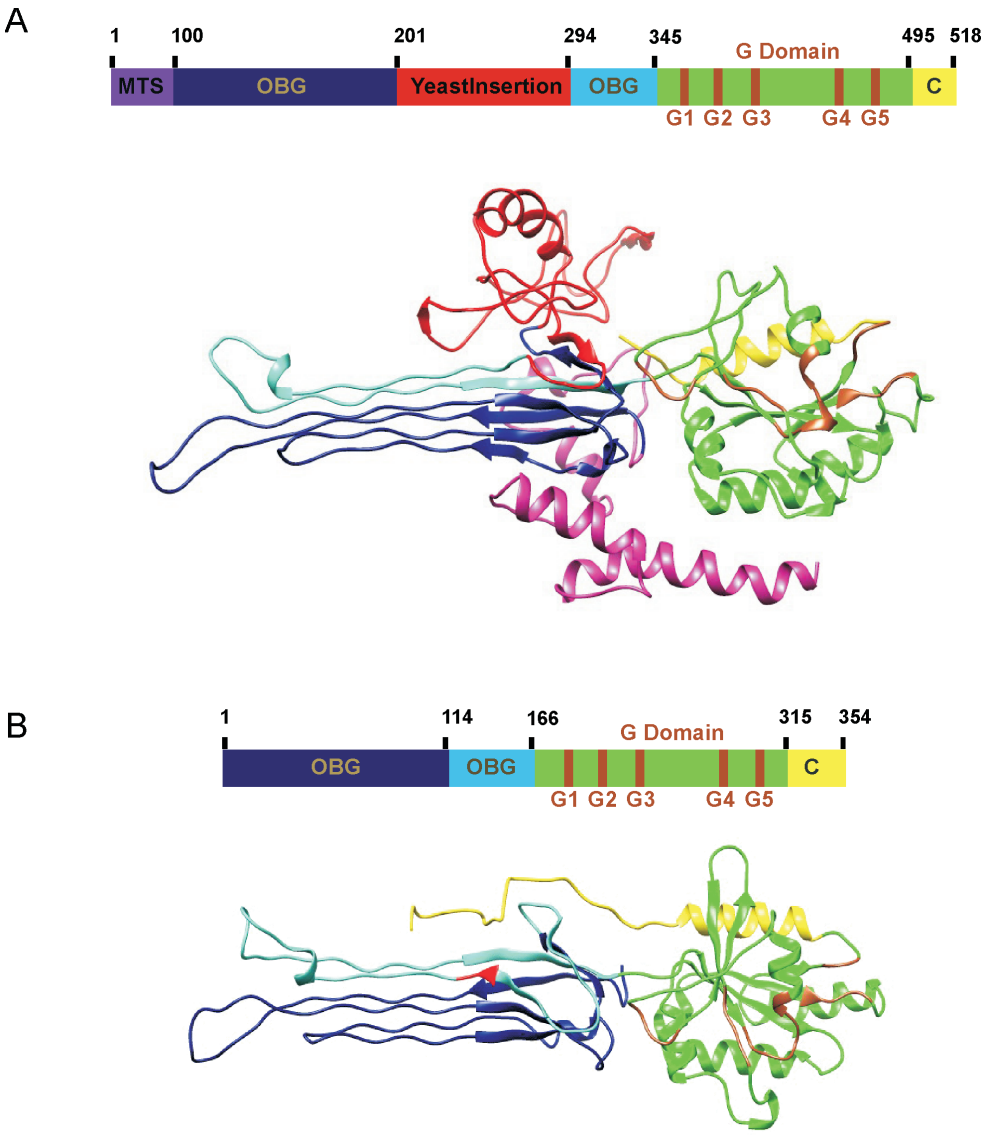

Figure 2: **Comparison of predicted structures of (A) Mtg2p and (B) *C. crescentus* Obg protein CgtA$_C$. The OBG fold (Buglino *et al.*, 2002) before and after the insertion sequence are rendered blue and turquoise, respectively. The insertion region in *MTG2* is rendered in red.The GTP binding domain is marked in green with motifs (G1-G5) rendered in brown. The C-terminal region is rendered in yellow. Schematic representation of various domains of Mtg2p and CgtA$_C$ are also included.**

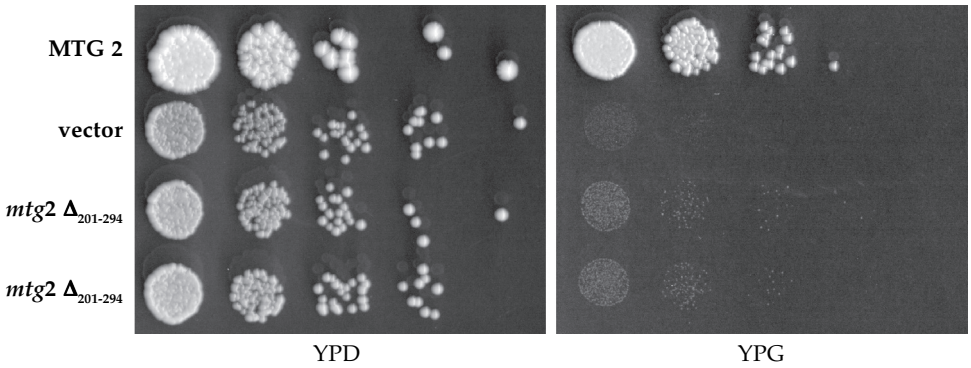

Figure 3: **Yeast specific insertion domain is essential for *MTG2* function *in vivo*. Complementation analysis of Mtg2p deleted for residues 201-294. Shown are serial dilutions of *Δmtg2ρ*$^+$cells harboring *mtg2*, empty vector, *mtg2*$_{Δ201-294}$ on YPD and YPG plates as indicated. Two independent colonies of cells harboring *mtg2*$_{Δ201-294}$ were analyzed.**

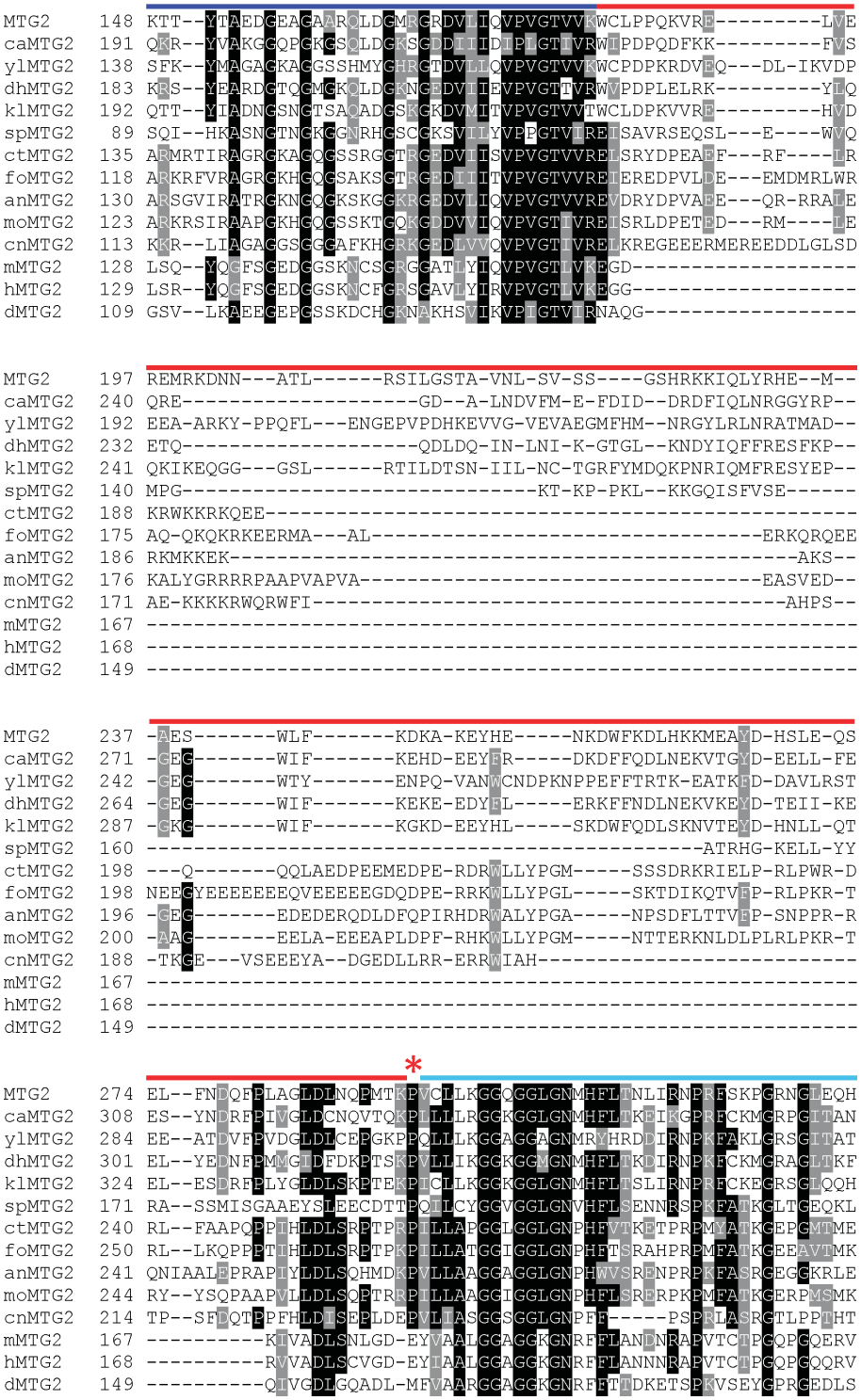

Figure 4: **Comparison of insertion domains in mitochondrial members of Obg proteins from indicated yeast. Identical amino acids are boxed in black whereas conserved amino acids are shaded. Sequences include Obg proteins from *Saccharomyces cerevisiae* (MTG2), *Candida albicans* (caMTG2), *Yarrowia lipolytica* (ylMTG2), *Debaryomyces hansenii* (dhMTG2), *Kluyveromyces lactis* (klMTG2), *Schizosaccharomyces pombe* (spMTG2), *Chaetomium thermophilum* (ctMTG2), *Fusarium oxysporum* (foMTG2), *Aspergillus nidulans* (anMTG2), *Magnaporthe oryzae* (moMTG2), *Cryptococcus neoformans* (cnMTG2).Junctions of the OBG fold were marked by including Obg proteins from *Mus musculus* (mMTG2), *Homo sapiens* (hMTG2), *Drosophila melanogaster* (dMTG2) in the comparison. The boundaries of OBG fold are indicated by blue and turquoise lines. The insertion domain is indicated by red line. A conserved proline residue at the junction of insertion domain and OBG fold is marked.**

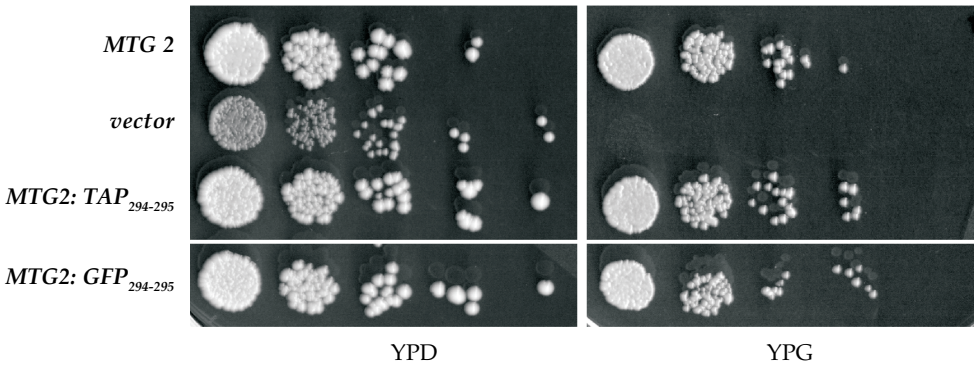

Figure 5: **Boundary between yeast specific domain and OBG fold is amenable to insertion for foreign DNA. Complementation analysis of *MTG2* tagged with Tandem affinity purification (TAP) tag or Green Fluorescent Protein (GFP) between amino acid residues 294-295. Shown are serial dilutions of *Δmtg2ρ*$^+$cells harboring empty *MTG2*, vector, *MTG2: TAP*$_{294-295}$ and *MTG2: GFP*$_{294-295}$ on YPD and YPG plates as indicated.**

sufficient to supports generalized mitochondrial translation whereas the CE domain is thought to aid assembly of mitochondrial translation product albeit by an unknown mechanism (Prestele *et al.*, 2009; Williams *et al.*, 2004). A similar role for the inserted domain comprising of residues 201-294 could be envisaged. Mtg2p has been shown to be an assembly factor for 54S mitochondrial large subunit (Datta *et al.*, 2005). Although a precise molecular mechanism of how Obg proteins influence ribosome biogenesis is absent, numerous non-exclusive speculative models have been proposed. Once such model predicts utilization of energy from GTP hydrolysis to recruit individual ribosomal proteins onto precursor molecules (Britton, 2009; Karbstein, 2007). A potential role for Mtg2p could involve recruitment of MRPs onto precursor 54S particles. MRPs recruited onto precursor 54S particles fall into two categories, those that are universally conserved and those that are specific to the yeast lineage (Amunts *et al.*, 2014). The recruitment of universally conserved MRP's could be influenced by domains of Mtg2p that have clear bacterial orthologues whereas the yeast specific insertion domain could be involved in recruitment of yeast specific MRPs. Consistent with this hypothesis, in the predicted Mtg2p structure, the yeast specific insertion folds into an autonomous domain without disrupting the overall structure of the OBG fold (Figure 2). In addition, insertion of relatively large protein sequences of TAP (187a.a.) or GFP (260 a.a.), having no sequence similarity within them, at the end of this yeast specific insertion region did not perturb Mtg2p function indicating that the insertion domain folds into an independent structure.

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Conflict of interest

The authors do not have any conflict of interest with the contents of this manuscript.

Abbreviations

mt, mitochondrial; MRP, mitochondrial ribosomal protein; TAP, Tandem affinity purification; YPD Yeast peptone dextrose; YPG, Yeast peptone glycerol.

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