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Validation of a *MGM1/OPA1* chimeric gene for functional analysis in yeast of mutations associated with dominant optic atrophy

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ABSTRACT

Mutations in *OPA1* are associated with DOA or DOA plus. Novel mutations in *OPA1* are periodically identified, but often the causative effect of the mutation is not demonstrated. A chimeric protein containing the N-terminal region of Mgm1, the yeast orthologue of OPA1, and the C-terminal region of OPA1 was constructed. This chimeric construct can be exploited to evaluate the pathogenicity of most of the missense mutations in *OPA1* as well as to determine whether the dominance of the mutation is due to haploinsufficiency or to gain of function.

1. Introduction

Dominant optic atrophy (DOA) is a mitochondrial disease characterized by mild to severe decrease in visual acuity, color vision deficiency and visual field defects, due to selective degeneration of retinal ganglion cells and optic nerve atrophy (Kjer, 1959; Lenaers et al., 2012). DOA is a genetically inherited disorder associated, in most cases, with mutations in the *OPA1* gene (Delettre et al., 2000; Alexander et al., 2000), which encodes the mitochondrial GTPase of the dynamin family OPA1, a highly conserved protein primarily involved in mitochondrial fusion events and in mtDNA maintenance. This protein is inserted in the inner mitochondrial membrane (IMM) and displays three highly conserved regions: a GTPase domain, a middle domain and a GTPase effector domain, involved in oligomerization and activation of dynamins. Eight OPA1 isoforms resulting from alternative splicing of exon 4, 4b and 5b are present in humans. These isoforms exhibit tissue-specific patterns whose specific function is still unknown (Landes et al., 2010). Each isoform can also undergo proteolytic cleavage generating long and short isoforms both of which are required for the IMM fusion and are involved in membrane tethering and GTPase activity respectively (Escobar-Henriques and Anton, 2013).

More than 200 pathogenic mutations spread throughout the entire *OPA1* gene have been identified so far (*OPA1* LSDB http://opa1.mitodyn.org). About 50% of these *OPA1* mutations (frame-shift and non-sense mutations, stop codons, splicing errors, or deletions/insertions) generate a haploinsufficiency situation where the mutated transcript is degraded by mRNA decay, thus reducing to 50% the amount of OPA1 protein (Schimpf et al., 2008). The remaining ones are missense mutations, which are mostly clustered in the GTPase domain and cause heterozygous amino acid substitutions thought to exert a severe dominant negative effect, because the mutated protein might interfere with and inhibit the wild-type protein (Amati-Bonneau et al., 2009). These latter mutations are often associated with a more severe syndromic disorder named "DOA-plus", which includes optic atrophy appearing in childhood, followed by chronic progressive external ophthalmoplegia (PEO), ataxia, sensorineural deafness, sensory-motor neuropathy, myopathy and mtDNA multiple deletions in adult life (Amati-Bonneau et al., 2008; Hudson et al., 2008; Yu-Wai-Man et al., 2010).

Abbreviations: CC, coiled coil; CMV, cytomegalovirus; COX, cytochrome c oxidoreductase; DOA, Dominant Optic Atrophy; DOA plus, Dominant Optic Atrophy plus phenotype; GED, GTPase effector domain; MPS, mitochondrial peptide signal; NCCR, NADH-cytochrome c oxidoreductase; PGK, phosphoglycerate kinase; RCR, rhomboid cleavage region; SQDR, succinate-coenzyme Q-2,6-Dichlorophenol Indophenol reductase; TETOff, tetracycline off promoter; TM, transmembrane region; wt, wild type.

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Studies carried out in fibroblasts and in lymphoblasts as well as in skeletal muscle from DOA patients have demonstrated that OPA1 has other crucial functions besides its role in mtDNA maintenance and mitochondrial fusion, i.e. *cristae* ultrastructure integrity and apoptosis regulation (Frezza et al., 2006), respiratory supercomplexes stability and energetic efficiency (Cogliati et al., 2013). However, even though skin fibroblasts are important in vitro cellular models for the study of DOA pathophysiology, using other in vivo models which are genetically easier to manipulate and are characterized by a strikingly different mutant phenotype from the wild-type one can be desirable.

To this purpose, the yeast *Saccharomyces cerevisiae*, whose *OPA1* orthologous gene is *MGM1* (Mitochondrial Genome Maintenance), can be considered a suitable model organism (Jones and Fangman, 1992) to test for pathogenicity of new *OPA1* variants identified in patients, as done previously for several mutations causing mitochondrial diseases. A number of cases are reported in which allegedly pathological human substitutions have been validated in yeast by using different strategies depending on the conservation of the residues involved and/or on the ability of the human wt gene to complement the function of the yeast gene (reviewed in Barrientos, 2003; Rinaldi et al., 2010; Baile and Claypool, 2013; Lodi et al., 2015; Lasserre et al., 2015). Although Mgm1 and OPA1 own equivalent functional domains, their amino acid sequences are weakly conserved, therefore only few pathological mutations can be introduced in *MGM1* and analyzed in yeast. Moreover in *S. cerevisiae OPA1* cannot substitute the *MGM1* gene. To overcome these problems chimeric proteins composed of the N-terminal region of Mgm1 fused with the catalytic region of OPA1 were constructed. One *MGM1*/*OPA1* chimera was able to complement the oxidative growth defect of the *S. cerevisiae mgm1* null mutant, thus validating this recombinant construct as a model for the study of the *OPA1* pathological mutations.

2. Materials and methods

2.1. Yeast strains and media

The yeast strains used in this work were W303-1A (*MATa leu2-3*, *trp1-1*, *can1-100*, *ura3-1*, *ade 2–1*, *his3-11*), W303-1B (*MATα leu2-3*, *trp1-1*, *can1-100*, *ura3-1*, *ade 2–1*, *his3-11*) and their isogenic strains *mgm1*::*KanR* harboring plasmid pFl38*MGM1* (see below). The diploid DW1.1 was obtained by crossing W303-1A *Δmgm1* and W303-1B *Δmgm1*.

Synthetic complete medium (SC) contained 6.9 g/l yeast nitrogen base without amino acids (Formedium), 1 g/l drop-out mix according to Kaiser et al. (1994). YP medium contained 0.5% yeast extract (Formedium) and 1% peptone (Formedium); YPA medium was YP 2X supplemented with 40 mg/ml adenine base (Formedium). Carbon sources were added as indicated in the text.

2.2. Plasmid construction

Constructs produced in this work were cloned in plasmids pFL38 (centromeric, *URA3* marker), pFL39 (centromeric, *TRP1* marker), pFL36 (centromeric, *LEU2 marker*) (Bonneaud et al., 1991) and YEplac112 (multicopy, *TRP1* marker) (Gietz and Sugino, 1988). All the primers employed in this study are reported in Supplementary Table 1.

The yeast *MGM1* gene and its upstream and downstream regions were first PCR amplified using genomic DNA of strain W303-1B as template and MGM1CFw and MGM1CRv as primers, then digested with *Sac*I and *Sal*I and cloned in pFL38, digested with the same enzymes (pFL38*MGM1*). *MGM1* was then subcloned in pFL39 and YEplac112 to obtain pFL39*MGM1* and YEPlac112*MGM1*.

MGM1/*OPA1* constructs, hereafter called *CHIM1-6*, each containing different portions of *MGM1* and *OPA1*, were obtained using the two-step overlap extension technique, a modification of the protocol used for site directed mutagenesis (Ho et al., 1989). For each chimera, the first PCR reaction was performed using the external forward primer MGM1CFw and the external reverse primer OPA1XhoRv, each in combination with reverse or forward internal primers respectively, designed in order to amplify different portions of *MGM1* (gradually longer) and *OPA1* (gradually shorter), as reported in Supplementary Table 1. Final chimeric constructs were obtained by using the different overlapping PCR fragments as templates and MGM1CFw and OPA1XhoRv as primers. The products, together with the whole *OPA1* coding region amplified with OPA1CFw and OPA1XhoRv, were then digested with *Sal*I and *Xho*I and cloned in a *Sal*I-*Xho*I-digested pFL39*MGM1* centromeric plasmid and in a *Sal*I-*Xho*I digested YEplac112*MGM1* multicopy plasmid, in each of which a *Xho*I site was introduced by site specific mutagenesis just after the stop codon of *MGM1* ORF.

The *CHIM3* construct was also cloned in pFL39PGK, pFL39TEToff and pFL36TEToff. pFL39PGK was obtained by subcloning the PGK promoter and the PGK terminator from plasmid pFL61 (Minet et al., 1992) digested with *Bam*HI and *Bgl*II in *Bam*HI-digested pFL39. pFL39TEToff and pFL36TEToff were obtained by subcloning the tTA transactivator under the CMV promoter, seven repeats of the tetO promoter upstream of the *CYC1* promoter and the *CYC1* terminator from plasmid pCM189 (Garì et al., 1997) digested with *Eco*RI and *Hind*III in *Eco*RI-*Hind*III-digested pFL39 and pFL36, respectively.

pFL39PGKCHIM3, pFL39TEToffCHIM3 and pFL36TEToffCHIM3 were obtained by PCR-amplification using pFL39*CHIM3* as template and MGMOPACNotFw and MGMOPACNotRv as primers. After digestion with *Not*I, the PCR fragments were cloned in the different plasmids and sequenced.

2.3. Construction of mutant alleles

chim3 mutant alleles were obtained by site-directed mutagenesis of a *CHIM3* gene fragment, using the overlap extension technique (Ho et al., 1989). In the first PCR, the forward primer TATACYC1Fw and specific reverse mutagenic primers were used. In the second PCR, specific forward mutagenic primers and the reverse primer MGMOPACNotRv were employed. The final mutagenized products were obtained by using the overlapping PCR fragments (obtained in the first and second PCR) as templates, with TATACYC1Fw and MGMOPACNotRv as external primers.

Each final product was then digested with *Not*I and cloned in *Not*I-digested pFL39TEToff, obtaining pFL39TEToff plasmid-borne $CHIM3^{I382M}$, $CHIM3^{R445H}$, $CHIM3^{K468E}$ and $CHIM3^{V903Gfs*3}$ mutant alleles.

2.4. Construction of strains harboring CHIM3 wt and mutant alleles

MGM1 was disrupted in W303-1A and W303-1B by one step gene disruption. At first both strains were transformed with pFL38*MGM1*. Then, the cassette *mgm1*::KanMX4 was amplified from the genomic DNA of BY4742 *Δmgm1* and inserted into both strains through high efficiency yeast transformation protocol (Gietz and Woods, 2002). Clones resistant to geneticin were PCR-amplified to verify the correctness of disruption. The W303-1A *Δmgm1*/pFL38*MGM1* strain was transformed with plasmids harboring the *TRP1* marker, and pFL38*MGM1* was lost through plasmid shuffling as previously reported (Boeke et al., 1987; Baruffini et al., 2010). To obtain diploid strains, W303-1A *Δmgm1*/pFL38*MGM1* was crossed with W303-1B *Δmgm1*, which had been transformed with pFL36TEToff. This diploid strain, selected on medium without leucine and uracil, was then transformed with *CHIM3* wt and mutant alleles on pFL39TEToff and subjected to plasmid shuffling to remove pFL38*MGM1*.

2.5. Complementation studies and measurement of extended mtDNA mutability

Oxidative growth was evaluated by spotting serial cell dilutions ($5x10^4$, $5x10^3$, $5x10^2$ and $5x10^2$ cell/spot), in a total volume of 5 μl, on