Deciphering OPA1 mutations pathogenicity by combined analysis of human, mouse and yeast cell models

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1. Introduction

Dominant optic atrophy (DOA) is one of the most common inherited optic neuropathies, characterized by degeneration of the retinal ganglion cells, leading to optic nerve atrophy and ultimately to blindness [1,2]. Most DOA patients harbor mutations in the OPA1 gene [3,4] encoding the mitochondrial GTPase OPA1, localized in the inner mitochondrial membrane [5]. This protein displays a mitochondrial-targeting sequence, a predicted transmembrane domain, followed by the three highly conserved regions: the GTPase, the middle and the GTPase effector domains. Eight OPA1 variants are present in humans, undergoing proteolytic cleavage by YME1L and OMA1 proteases [6] and generating a mixture of long and short isoforms (l- and s-forms) anchored to the inner membrane or soluble, respectively [7,8].

Currently, over 370 OPA1 gene variants have been identified, of which 65% is considered pathogenic. About 50% of the pathogenic variants cause the premature truncation of the open reading frame [9], supporting haploinsufficiency as the main pathological mechanism of the disease. The missense variants, mostly clustered in the GTPase domain and envisaged to exert a dominant-negative effect [10], are associated with high risk to developing the severe multisystem disorder DOA “plus” characterized by additional extraocular features, including sensorineural deafness, ataxia, myopathy, chronic progressive external ophthalmoplegia, peripheral neuropathy, multiple sclerosis-like features and parkinsonism/dementia [11–15]. More recently, severe clinical phenotypes, ranging from Behr to Leigh syndromes, have been associated with compound heterozygosity of OPA1 mutations, frequently in combination with the I382M hypomorphic allele, or homozygous OPA1 mutations qualifying as recessive forms [16–20].

Different degrees of mitochondrial network fragmentation and cristae disorganization have been reported in fibroblasts [11,21–24] and skeletal muscle from DOA patients [11], as well as in DOA mouse models [25–27].

Phosphorus magnetic resonance spectroscopy revealed in vivo
defective oxidative phosphorylation (OXPHOS) with reduced ATP production in muscle of DOA patients with several nonsense and mis-sense OPA1 mutations [28,29]. Dysfunctions of the energetic metabolism, i.e. decreased ATP synthesis rate, respiratory complexes activities, oxygen consumption and membrane potential were also documented in fibroblasts and lymphoblasts of patients carrying different OPA1 mutations [21,23,24,30].

Mitochondrial DNA (mtDNA) depletion was never reported in fibroblasts or skeletal muscles of DOA and DOA “plus” patients [11,12,24,31], showing rather increased mtDNA copy number in COX-negative fibers [13]. Conversely, mtDNA deletions, initially associated with the severe DOA “plus” phenotype only [11,31], were subsequently reported in skeletal muscles of the majority of patients harboring OPA1 mutations, even in those with non-syndromic DOA [29]. Only recently, the identification of families with truly recessive OPA1 mutations and affected with a multisystem disorder, documented the occurrence of mtDNA depletion in skeletal muscle [18].

It is noteworthy that primary skin fibroblasts derived from patients bearing OPA1 mutations represent an important cell model for studying DOA pathophysiology, even if they often reveal a rather mild phenotype, likely as a consequence of the hemizygous transmission of DOA and the type/position of the mutation. Therefore, cell models avoiding the partial compensatory effect of the wild-type allele would enhance the defective phenotypic profile of the pathogenic OPA1 mutant allele. To this end, we developed two different cell models. The first is the Mgm1/OPA1 chimera yeast model, that has been previously described in detail [32]. The second takes advantage of Opa1−/− mouse embryonic fibroblasts (MEFs), where human OPA1 isoform 1 (ISO1) bearing OPA1 mutations was expressed. The strength of these two models is that they are designed to provide complementary and non-overlapping information, thus allowing for a quick and comprehensive analysis of OPA1 mutations pathophysiology. Here we present the detailed molecular and functional characterization of fibroblasts, MEFs and Mgm1/OPA1 chimeras, demonstrating a faithful genotype/phenotype correlation, associating the DOA “plus” phenotype with a combinatorial effect of mitochondrial network fragmentation and mtDNA depletion, and validating a novel OPA1 mutation.

2. Materials and methods

2.1. Plasmid construction and retroviral transduction

Plasmid expressing the OPA1 isoforms 1 [7] was further mutagenized (I382M and D603H) and cloned into the pMSCV-puro vector, whereas the plasmids expressing isoform 1 bearing the mutations G439V and R445H were previously described [33]. Retrovirus production and infection were performed as described previously [34]. Plasmid (6 μg/experiment) transfection of mouse embryonic fibroblasts (MEFs) was performed with Lipofectamine 3000 (Life Technologies) following the manufacturer’s instructions.

2.2. Cells and culture conditions

Skin fibroblasts were derived, following informed consent, from five healthy donors, two DOA patients from two unrelated families with the I382M mutation, one patient with the G439 V mutation, two related patients with the R445H mutation and two related patients with the D603H mutation. OPA1 gene localization of these mutations is reported in Table 1. All mutations were heterozygous and strictly co-segregated with individuals affected by optic atrophy in families with autosomal dominant inheritance.

The range of age for patients’ fibroblasts is 24–59 years and for controls’ fibroblasts is 24–60 years, with 3 men and 2 women. Matching healthy donors with DOA patients for age and sex, the behavior of the five controls used was very similar and we decided to pull the controls together.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Summary of OPA1 mutations with amino acid change, clinical phenotype and pathogenetic prediction (CADD phred &gt; 20 are considered as pathogenic variants [52]).</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DNA mutation</strong></td>
<td><strong>Protein domain</strong></td>
</tr>
<tr>
<td>variant 1</td>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
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<td>c.2729T &gt; A</td>
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</table>

Fibroblasts and MEFs were grown in DMEM containing 25 mM glucose supplemented with 10% fetal bovine serum (FBS, South America, Gibco, Life Technologies), 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin, in an incubator with a humidified atmosphere of 5% CO2 at 37 °C. For some experiments, cells were grown in glucose-free DMEM supplemented with 5 mM galactose, 5 mM sodium pyruvate and 5% FBS (DMEM-galactose).

2.3. Cellular ATP content

The ATP content of cells in DMEM and during incubation in DMEM-galactose was measured by using the luciferin/luciferase assay, as previously described [35]. For statistical analysis, the Dunnett’s multiple comparison test was used.

2.4. Mitochondrial network morphology

Mitochondrial network morphology was analyzed as previously described in [36]. For statistical analysis, the Dunnett’s multiple comparison test was used.

2.5. mtDNA content

Quantification of human mtDNA copy number relative to nuclear DNA (nDNA) was performed, for both fibroblasts and muscle tissue, as previously described [37]. Quantification of mouse mtDNA copy number relative to nuclear DNA (nDNA) was carried out amplifying both mt-Nd1 and β-Globin. Quantification was performed using the ΔΔ Ct(1) method. Primer sequences are reported in supplementary materials. For statistical analysis, the Dunnett’s multiple comparison test was used.

2.6. Oxygen consumption rate

The OCR was measured in XFe24 Extracellular Flux Analyzer (Seahorse Biosciences). Oxygen concentration were measured under basal condition and after injection of 1 μM oligomycin, 1 μM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) or 5 μM carbonyl cyanide m-chlorophenyl hydradrazine (CCCP), 1 μM rotenone and 1 μM antimycin A. The OCR values (pmoles O2/min) were normalized for protein content of each well, determined by the SRB assay. For statistical analysis, the Dunnett’s multiple comparison test was used.

2.7. Western blotting

Cell lysates were prepared as previously described [24]. Aliquots were separated by 8% SDS-PAGE and transferred onto nitrocellulose...
membranes (Bio-Rad). The membranes were incubated overnight at 4 °C with the primary antibodies, then visualized using horseradish peroxidase-conjugated secondary antibodies. The chemiluminescence signals were revealed using an ECL Western blotting kit and measured with Gel Logic 1500 Imaging System, Biosense. For statistical analysis, the Dunnett’s multiple comparison test was used.

2.8. Respiratory supercomplexes and complex V analysis

Supercomplexes were separated by Blue Native-PAGE and analyzed as previously described [36]. For statistical analysis, the Dunnett’s multiple comparison test was used.

2.9. cDNA Sequencing

Total RNA was extracted from fibroblasts using the Pure Link RNA mini kit (Ambion) and treated with DNAseI enzyme (Sigma Aldrich). 1 μg of RNA was reverse transcribed by SuperScript VILO cDNA synthesis kit and analyzed by Sanger Sequencing, as previously reported [38].

2.10. Yeast strains and media

The yeast strains used in this work were W303–1A Δmgm1 (MATa leu2–3, trp1–1, can1–100, ura3–1, ade2–1, his3–11 mgm1::KanR), W303–1B Δmgm1 (MATa leu2–3, trp1–1, can1–100, ura3–1, ade 2–1, his3–11 mgm1::KanR). The diploid DW1.1 was obtained by crossing W303–1A Δmgm1 and W303–1B Δmgm1 [32]. 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. [39]. YP medium contained 0.5% yeast extract (Formedium) and 1% peptone (Formedium); YPA medium was YP 2× supplemented with 40 mg/ml adenine base (Formedium). Carbon sources were added as indicated in the text.

2.11. Plasmids and construction of strains harboring CHIM3 wt and mutant alleles

The plasmids used in this works are pFL38TETo, pFL39TETo, pFL36TETo, pFL38MGGM1 and pFL39MGGM1 [32]. chim3 mutant alleles were obtained by site-directed mutagenesis of a CHIM3 gene fragment, using the overlap extension technique [40]. In the first PCR, the forward primer TATACYC1Fw and specific reverse mutagenic primers were used (see primers list in the supplementary materials). 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 as templates, with TATACYC1Fw and MGMOPACNotRv as external primers. Each final product was then digested with NotI and cloned in NotI-digested pFL39TEToR, obtaining pFL39TEToR plasmid-borne CHIM3ΔSS900N, CHIM3Δ2000E, CHIM3Δ331P, CHIM3ΔG439V, CHIM3Δ449P, CHIM3Δ534ER, CHIM3Δ358P, CHIM3Δ660IR, CHIM3Δ788R and CHIM3Δ916D mutant alleles. Mgm1 mutant alleles were obtained in the same way, using the forward primer MFM1FwSaIl and the reverse primer MGM1RvSaI together with mutagenic primers reported in supplementary materials. Mutant and wt CHIM3 alleles were introduced in the Δmgm1 strain through the plasmid shuffling technique as previously reported [41].

2.12. Complementation studies in yeast

Oxidative growth was evaluated by spotting serial cell dilutions of haploid strains (5 × 10^4, 5 × 10^3, 5 × 10^2 and 5 × 10^1 cell/spot) in a total volume of 5 μl on SC medium, supplemented with either 2% glucose or 2% glycerol after a 5-days incubation at 28 °C. Diploid strains were diluted to an initial concentration of 0.08 OD/ml and then four 10-fold serial dilutions were made. A total volume of 5 μl was spotted on solid SC medium, supplemented with 2% glucose or 1.5% glycerol-1.5% ethanol and the growth was evaluated after a 7-days incubation at 28 °C.

To measure the respiratory activity, diploid strains were pre-grown in SC supplemented with 2% galactose for 48 h to limit the growth of petite cells. Cells were inoculated at a final concentration of 0.08 OD/ml in SC supplemented with 0.6% glucose and grown until glucose was exhausted for approximately 20 h. The OCR was measured on whole cells using a Clark-type oxygen electrode (Oxygraph System Hansatech Instruments England) with 1 ml of air-saturated respiration buffer (0.1 M phthalate–KOH, pH 5.0), 10 mM glycerol at 30 °C. The reaction started by addition of 20 mg of wet-weight cells, as described previously [42,43]. For statistical analysis, the Dunnett’s multiple comparison test was used.

2.13. Western blot analysis in yeast

Total proteins from 4.8 OD (for haploid strains) and 3.6 OD (for diploid strains) of cells grown in SC supplemented with 0.6% glucose
Fig. 2. Energetic characterization of OPA1 mutated fibroblasts.

(A) Cellular ATP levels after incubation for 24 and 48 h in DMEM-galactose. Data, expressed as % of ATP content at time = 0, are means ± SEM of WT (n = 10), mutants (n = 6) except for G439V (n = 3). (B) OCR of fibroblasts in DMEM-glucose, expressed as pmol O₂/min normalized for protein content, under basal conditions and after injection of oligomycin (O), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP; F), rotenone (R) and antimycin A (AA). (C) Basal, ATP-linked, maximal respiration and spare respiratory capacity. (D) Ratio between maximal and ATP-linked respiration. (E) Cell respiratory control ratio (RCR). (B-E) Data are means ± SEM of WT (n = 10), mutants (n = 6) except for G439V (n = 5). (F) OCR of fibroblasts in DMEM-galactose (24 h). The measure was performed in DMEM-galactose medium. (G) Basal, ATP-linked, maximal respiration and spare respiratory capacity. (H) Ratio between maximal and ATP-linked respiration. (F–H) Data are means ± SD of WT and mutants (n = 3). (A–H) For E382M, R445H and D603H mutations, data are means of two patients with the same mutation. (I) Cl IGA and western blot analysis of supercomplexes after BN-PAGE of digitonin-solubilized mitochondria. SDHA (CII) was used as a loading control. Supercomplexes composition and single complexes are indicated. SDS-PAGE of SDHA and VDAC used as a loading control. A representative experiment out of three is shown. Bars indicate that one lane was removed from the blot. (J) Densitometric analysis of Cl alone and Cl-containing supercomplexes. Data, expressed as % of WT cells ratio, are means ± SD of three independent experiments.

In all panels * and ** denote values significantly different from the WT (p < 0.05 and p < 0.01, respectively) using Dunnett’s multiple comparison test.

until exhaustion were extracted with the trichloroacetic acid (TCA) method by chilling the cells supplemented with 120 mM NaOH, 0.5% β-mercaptoethanol, 650 μM PMSF and 25% TCA on ice, then re-suspending the proteins in 60 μl of Laemmli sample buffer at pH 6.8. 10 μl of such suspension were separated by 8% SDS-PAGE, and electroblotted onto nitrocellulose filters which were then incubated with anti-OPA1 and anti-VDAC antibodies. After incubation with anti-mouse secondary antibodies, the chemiluminescence signals were revealed using by ECL Western blotting substrate and measured with Imagequant Software (GE Healthcare).

2.14. Mitochondrial morphology in yeast

Haploid and diploid strains carrying CHIM3 or chim3 mutant alleles were transformed with pYEF1mtGFP [32]. Cells were pre-grown in SC medium supplemented with 2% glucose. After 24 h, the cells were transferred to SC medium supplemented with 0.15% glucose and 2% galactose in order to induce the expression of mtGFP, until the mid-log phase was reached. Once adjusted to 1 OD/ml, the cells were observed with a Zeiss Observer 2.1 fluorescence microscope using a 630 × magnification and images captured using an Axiosvision Rel 4.8 software. Statistical analysis was performed using a χ² test on a 2 × 2 contingency table comparing the number of cells with filamentous mitochondria vs the number of cells with giant or fragmented mitochondria.

2.15. Template search, sequence alignments, and OPA1 homology modeling

Template search was performed using the HHsearch method employed by the HHpred server (doi:https://doi.org/10.1093/nar/gki408). The server performs up to eight iterative PSI-BLAST (doi:https://doi.org/10.1093/nar/25.17.3389) searches through the non-redundant (nr) database from the NCBI. Using the final target alignment a profile hidden Markov model (HMM) (doi:https://doi.org/10.1006/jmbi.1994.1104; doi:https://doi.org/10.1093/bioinformatics/14.9.755) is calculated. Homologous templates are identified by searching through a weekly updated database containing HMMs for a representative subset of PDB sequences. Finally, HHsearch ranks database matches by the probability of the match to be homologous to the target sequence. This is useful to distinguish homologous from non-homologous matches. Excluding PDB structures that were not solved by using X-ray crystallography, HHpred identified the human myxovirus resistance protein 1 (MxA), an interferon-induced dynamin-like GTPase and the human Dynamin 1-like protein (DNM1L) as the best templates for OPA1.

The target and template sequences were realigned using the Promals3D server (doi:https://doi.org/10.1093/nar/gkn072) (see Supplementary Information for the results of the alignment). The obtained alignment was then used to calculate 100 models of OPA1 structure in the “open” conformation using the available crystal structures of human MxA [PDB code: 3SZR (doi:https://doi.org/10.1016/j.immuni.2011.07.012)] and human DNM1L [PDB code: 4BEJ (doi:https://doi.org/10.1038/embr.2013.74)], using the Modeller 9.14 software (doi:https://doi.org/10.1006/jmbi.1993.1626). The GDP molecule found in the human DNM1L structure was included in the modeling procedure. The best model was selected using the DOPE potential function built into Modeller (doi:https://doi.org/10.1110/ps.062416606). A loop optimization routine was used to refine the regions that showed higher than average energy as calculated using the DOPE potential function. The final model was geometry optimized in the gas phase by using the UCSF Chimera (doi:https://doi.org/10.1002/jcc.20084), the AMBER ff14SB force field (doi:https://doi.org/10.1021/acs.jctc.5b00255), and the AM1-BCC charges (doi:https://doi.org/10.1002/jcc.10128). The stereochemical quality of the model structure was established using ProCheck (doi:https://doi.org/10.1107/S002188989900944). The results of this analysis confirm the reliability of the model structure.

The template structure used to calculate the OPA1 model structure in the “closed” conformation was prepared by dividing the model in the “open” conformations in two parts, one formed by the GTPase domain of the other formed by the helical domain. The two parts of the OPA1 model in the “open” conformation was then superimposed to the corresponding domains of the known structure of the bacterial dynamin like protein from Nostoc punctiforme [PDB code: 2J68 (doi:https://doi.org/10.1038/nature05312)]. This template structure was then used to calculate the model of OPA1 in the “closed” conformation, following the same protocol described above.

2.16. Reagents

Antibodies: DRP1, MFN1, MFN2 (Abnova); NDUFA9, UQCRC2, α subunits CV, actin (Abcam); COX IV, SDHA (ThermoFisher Scientific); OPA1 (BD Biosciences); tubulin (Sigma-Aldrich); VDAC (Biovision); subunits CV, actin (Abcam); COX IV, SDHA (ThermoFisher Scientific); OPA1 (BD Biosciences); tubulin (Sigma-Aldrich); VDAC (Biovision); horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch or Alexafluor). ECL western blotting kit from Biorad or Thermo Scientific Pierce. The other reagents from Sigma-Aldrich.

3. Results

3.1. Molecular and biochemical profile of fibroblasts bearing OPA1 mutations

We have investigated fibroblasts derived from seven patients carrying four different OPA1 missense mutations and five healthy donors. Three of these mutations were selected on the basis of their known clinical phenotypes, ranging from non-penetrant or very mild pure optic atrophy (I382M), to severe syndromic forms including severe visual loss, is here reported and fully characterized for the first time (Supplementary Table 1 for description of patients’ clinical phenotypes). The fourth mutation, D603H, clinically expressed as pure DOA with severe visual loss, is here reported and fully characterized for the first time (Supplementary Table 1 for description of patients’ clinical phenotypes). The other formed by the helical domain. The two parts of the OPA1 model in the “open” conformation was then superimposed to the corresponding domains of the known structure of the bacterial dynamin like protein from Nostoc punctiforme [PDB code: 2J68 (doi:https://doi.org/10.1038/nature05312)]. This template structure was then used to calculate the model of OPA1 in the “closed” conformation, following the same protocol described above.

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Fig. 3. mtDNA content and mitochondrial network morphology of OPA1 mutated fibroblasts.  
(A) mtDNA copy number. Data are means ± SEM of WT (n = 10), mutants (n = 6) except for G439 V (n = 3).  
(B) Representative images captured by fluorescence microscopy of mitochondrial network morphology of fibroblasts labeled with Mitotracker Red. Cell were incubated in DMEM-glucose (C) or for 24 h in DMEM-galactose (D); 40 cells for each cell lines in duplicate (2 independent experiments) has been analyzed for controls (5 cell lines, 400 total cells) for each condition; 60 cells for each cell lines in triplicate (2 independent experiments) has been analyzed for mutants (7 cell lines; 180 total cells for the G439 V mutation from one patient and 360 total cells for each of the other three mutations from six patients) for each condition. Cells were scored in three categories on the basis of mitochondrial network morphologies: cells with filamentous and interconnected network (filamentous), cells with short filamentous mitochondria (intermediate) and cells with fragmented mitochondria (fragmented). (A, C-D) For I382M, R445H and D603H mutations, data are the means of two patients with the same mutation. Data are means ± SEM of three independent experiments. In all panels *, ** and *** denote values significantly different from the WT (p < 0.05, p < 0.01 and p < 0.001, respectively) using Dunnett's multiple comparison test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Table 2

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<th>RCS</th>
<th>Network</th>
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</tr>
<tr>
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<td>+80%*</td>
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<td>No</td>
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<td>2</td>
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<td>No</td>
<td>−100%*</td>
<td>No</td>
<td>No</td>
<td>10%</td>
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</table>

* Percent increase/decrease after 24 h DMEM-galactose vs control fibroblasts.

† OCR in DMEM-glucose.

‡ Percentage of fibroblasts with fragmented mitochondria in DMEM-galactose.

* p < 0.05

** p < 0.01

fibroblasts and then in other two cellular models generated “ad hoc”.

The expression levels of MFN1, MFN2 and DRP1, the main proteins involved in the machinery of mitochondrial fusion and fission, were similar in control and DOA patients, whereas the amount of OPA1 was reduced in fibroblasts with the I382M, R445H and D603H mutations and slightly increased in G439 V fibroblasts (Fig. 1A–B). Sequencing of retro-transcribed OPA1 mRNA in the same fibroblasts revealed the presence of both wild-type and mutated alleles (Fig. S1A), indicating that both the proteins were synthesized. Quantification of the relative amount of l- and s-forms revealed an increased l−/s-forms ratio for the G439 V mutation only (Fig. 1C).

We then studied the energetic competence of DOA patients’ fibroblasts, by evaluating initially the cellular ATP content during incubation in glucose-free DMEM containing galactose, a condition known to force cells to rely on OXPHOS for ATP synthesis. After 24 h and 48 h in DMEM-galactose, the ATP levels of controls markedly increased (+60 ± 15% and +40 ± 17%, respectively), as a consequence of the shift from glycolytic to oxidative metabolism. Differently, all fibroblasts bearing OPA1 mutations were unable to restore the ATP content, being the differences from controls statistically significant at 24 h only, when cells have not yet adjusted their metabolism to the change of carbohydrate source (Fig. 2A). The same trend was evident by expressing the ATP values as nmol/mg of protein (Fig. S1B). A similar behavior was previously reported for DOA fibroblasts harboring OPA1 mutations leading to haploinsufficiency [24]. The oxygen consumption rate (OCR) was slightly reduced, without reaching statistical significance, in fibroblasts bearing the G439 V mutation only (Fig. 2B–C), although the maximal/ATP-linked ratio and the respiratory control ratio (RCR) were similar in all cell lines (Fig. 2D–E). In DMEM-galactose the respiration was increased in all cell lines, but mutant fibroblasts were less efficient than controls, as evidenced by the non-significant reduction of OCR traces and maximal/ATP-linked ratio (Fig. 2F–H).

Semi-quantitative evaluation of the Complex I (Cl) in gel activity (Cl-IGA) and its densitometric analysis revealed that the amounts of isolated Cl and of Cl + III2 supercomplex were reduced in mutated fibroblasts compared to controls, except for the D603H mutation. The level of the most abundant Cl + III2 + IV supercomplex was similar to controls in all mutants, except for the D603H. The assembly of CV monomer was not compromised by any of the OPA1 mutations (Fig. 2G–I).

All together these results indicate that despite mutants’ inability to increase the ATP content and respiration during the shift from glycolytic to oxidative metabolism, the supramolecular organization ofOXPHOS complexes is not dramatically affected by OPA1 mutations in fibroblasts.

Considering the OPA1 involvement in mtDNA maintenance [11,44], we also determined the mtDNA content. As reported in Fig. 3A, in fibroblasts with the I382M and D603H mutations the mtDNA copy number was similar to controls. Differently, the mtDNA amount was significantly reduced in R445H mutants and increased in fibroblasts with the G439 V (Figs. 3A and S1C). The separate analysis of the two fibroblast cell lines carrying the R445H mutation revealed similar OPA1 levels (Fig. S1D) but different mtDNA content (Fig. S1E). Indeed, only the fibroblasts derived from patient II exhibited a marked reduction of mtDNA copy number, as also confirmed by analysis in the muscle biopsy (Fig. S1F). The mtDNA amount in the muscle biopsy of the G439 V patient was normal (Fig. S1F).

The mitochondrial network morphology was then investigated by fluorescence microscopy. Representative images of fibroblasts incubated in DMEM-glucose or DMEM-galactose are reported in Fig. 3B. Quantitative analysis of mitochondrial morphology of fibroblasts incubated in DMEM-glucose showed an almost complete filamentous mitochondrial network in controls and in cells with the I382M, whereas G439 V and R445H mutations were associated with a slight increase of intermediate mitochondria. The D603H mutation presented a significant reduction of filamentous mitochondria and increase of intermediate mitochondria (Fig. 3C). In DMEM-galactose, the percentage of cells with fragmented mitochondria, which remained negligible in control fibroblasts, was variously increased by the presence of each OPA1 mutation, being statistically significant for the G439 V and R445H mutations (60% and 40%, respectively, Fig. 3D). Again, D603H mutation presented a significant reduction of filamentous mitochondria and an increase of intermediate mitochondria (Fig. 3C). The results are summarized in Table 2.

All together these results underline the difficulty of studying the pathogenicity of an OPA1 mutation and its molecular mechanism in patient fibroblasts that always present mild phenotypes, even when bearing severe mutations associated to DOA “plus”.

3.2. Molecular and biochemical profile of MEF bearing OPA1 mutations

We took advantage of Opa1−/− MEFs to express the OPA1 isoform 1 bearing each of the same four mutations analyzed in patients’ fibroblasts. This cell model, previously characterized by our group [36], allows to examine the specific effect of each mutation in the same nuclear background and without the confounder of the wt allele.

Western blot analysis demonstrated that the balance of OPA1 l- and s-forms compared to ISO1 as well as the level of the other mitochondrial shaping proteins was not influenced by the different mutations (Fig. 4A–B).

Because of the faster replication rate of MEFs compared to fibroblasts, the ATP content in DMEM-galactose was measured after 16 h and 24 h of incubation. After 16 h, MEFs bearing the G439 V and R445H mutations exhibited a significant reduction of ATP levels, similar to those of Opa1−/− MEFs, whereas MEFs with the other OPA1 mutations were similar to wt (Fig. 4C and S2A). The OCR traces are reported in Fig. 4D. Variable respiratory deficits were apparent in OPA1 mutated MEFs (Fig. 4D–E), ranging from a slight, not significant reduction of the maximal/ATP-linked respiration ratio apparent for I382M and D603H MEFs, to values similar to Opa1−/− MEFs in R445H and G439 V MEFs (Fig. F). These two mutations presented also a non-significant reduction of OCR traces and maximal/ATP-linked ratio (Fig. 4H).

The western blot analysis of OXPHOS subunits revealed reduced levels of NDUF9 (Cl), UQCRC2 (CIII) and COX IV (CIV) subunits in G439 V and R445H MEFs (Fig. 5A–B). Concordantly, the semi-quantitative evaluation of Cl-IGA and its densitometric analysis showed a reduction of isolated Cl, Cl + III2 and Cl + III2 + IV supercomplexes in G439 V and R445H MEFs compared to wt or ISO1, whereas D603H and I382M mutant MEFs displayed almost normal supramolecular assembly. Noticeably, the band at higher molecular weight corresponding to the Cl + III2 + IV supercomplex was almost absent in MEFs bearing mutations in the GTPase domain (Fig. 5C–D).

Immunodetection of CV revealed in G439 V and R445H MEFs, similarly to Opa1−/− MEFs, a marked reduction of the band corresponding to
the assembled monomer (F₁-F₃) and the appearance of two bands at lower molecular weight corresponding to free F₁, indicating the presence of partially disassembled CV sub-complexes (Fig. 5C). These results underline a severe energetic defect associated with G439V and R445H mutations, whereas D603H only induces a slight energetic dysfunction, completely absent in I382M cells.

It is noteworthy that the G439V and R445H mutations were associated with decreased mtDNA content, whereas the other two mutations exhibited values similar to those of wt and ISO1 MEFs (Fig. 6A).

Representative images of mitochondrial network of MEFs bearing OPA1 mutations are reported in Fig. 6B. Quantification of network morphology showed that MEFs with the I382M mutation presented a distribution similar to ISO1 MEFs, whereas the D603H induced both a significant increase in the percentage of cells with fragmented network and reduction of those with filamentous network. MEFs bearing the other two mutations exhibited the complete fragmentation of the...

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**Fig. 4.** Biochemical characterization of OPA1 mutated MEFs.
(A) Western blot of OPA1, MFN1, MFN2 and DRP1 expression levels; tubulin was used as a loading control. A representative blot out of three is shown for MFN1, MFN2 and DRP1. A representative blot out of eight is shown for OPA1. (B) Densitometric analysis of OPA1 l−/−/s-forms ratio. Data, expressed as % of ISO1 cells ratio, are means ± SEM (n = 8). (C) Cellular ATP levels. Data are means ± SEM of WT and ISO1 (n = 4) and all the other cell lines (n = 3). (D) OCR of OPA1 mutated MEFs, after the injection of oligomycin (O), carbonyl cyanide m-chlorophenyl hydrazone (CCCP; C), rotenone (R) and antimycin A (AA). (E) Basal, ATP-linked, maximal respiration and spare respiratory capacity. (F) Ratio between maximal and ATP-linked respiration. (G) Cell respiratory control ratio (RCR). (H) OCR and extracellular acidification rate (ECAR) ratio. (D-H) Data are means ± SD of Opa1−/− (n = 2) and all the other cell lines (n = 3). * and ** denote values significantly different from the WT (p < 0.05 and p < 0.01, respectively) using Dunnett's multiple comparison test.
mitochondrial network (Fig. 6C). The results are summarized in Table 3.

Overall these results evidenced that I382M mutation, associated with asymptomatic/very mild DOA, did not perturb mitochondrial morphology and mtDNA content, whereas D603H mutation, linked to pure DOA with severe visual loss, promoted mitochondrial fragmentation without any mtDNA reduction. Finally, G439V and R445H mutations, related with DOA “plus”, induced both mitochondrial fragmentation and significant mtDNA content decrease.

To verify this emerging relationship between mtDNA and mitochondrial fragmentation as indicator of pure DOA or DOA “plus” outcome, we decided to examine three other mutations: R290Q (DOA “plus”; Supplementary Table 1 for description of clinical phenotypes), S545R (pure DOA with severe optic atrophy [45] or DOA “plus” [11,31]) and Q785R (pure DOA [38]) (see positions within the OPA1 protein and other details in Table 1).
The expression of OPA1, MFN1, MFN2 and DRP1 proteins was not influenced by these three mutations (Fig. S2B). The mutation R290Q presented a significant decrease of mtDNA content (−30 ± 6%) and completely fragmented mitochondrial network (Fig. S2C–E), whereas the S545R mutation exhibited a non-significant decline (−25 ± 10%) in presence of complete mitochondrial fragmentation (Fig. S2C–E). The Q785R mutation did not change the mtDNA amount, still presenting a substantial increase in the percentage of cells with fragmented network (+60 ± 4%) (Fig. S2C–E). Thus, the Q785R mutation acts as the D603H, both associated to pure DOA, the R290Q behaves as the DOA "plus" related G439V and R445H mutations, and the S545R has an borderline behavior between pure DOA with severe optic atrophy and DOA "plus".

3.3. Phenotypic profile of yeast strain harboring mutations in OPA1

The yeast *S. cerevisiae* has been extensively used as an in vivo model to study and validate the pathogenicity of mutations in genes involved in mitochondrial diseases, thanks to its ability to grow in absence of OXPHOS activity. Although yeast Mgm1 and OPA1 are orthologous, the sequence of the two proteins is poorly conserved, and the whole OPA1 cannot complement the deletion of *MGM1* [32].

In order to validate OPA1 mutations in non-conserved residues, we have previously generated an *MGM1/OPA1* chimeric construct (called CHIM3), encoding a protein composed of the N-terminal region of Mgm1, comprising the sequence required for mitochondrial import, and the transmembrane domain, fused to the catalytic region of OPA1, containing the GTPase, the middle and the GED domains [32].

To acquire further insights on the consequences of the four missense mutations studied in fibroblasts and in MEFs on the OXPHOS
Fig. 7. Characterization of I382M, G439V, R445H and D603H mutations in yeast.

(A) Phenotypic analysis of haploid strains. Serial cell dilutions (5 × 10⁴, 5 × 10³, 5 × 10² and 5 × 10¹ cell/spot) of W303-1B Δmgm1 strain transformed with CHIM3 wild type or mutant alleles were spotted on YP medium, supplemented with either 2% glucose and 2% glycerol. Pictures were taken after a 3-days incubation at 28 °C. A representative experiment out of three independent clones is shown. (B) Western Blot analysis of OPA1 expression level. VDAC was used as loading control. A representative experiment out of two is shown. Bars indicate that one lane was removed from the blot. (C) Ratio between the l-form and the s-form normalized to the ratio observed in CHIM3. Data are means ± SD. (D) Representative example of the mitochondrial morphotypes in the investigated yeast strains. (E) Mitochondrial morphotypes percentage in haploid strains. 148 to 328 cells for each strain were observed at the microscope as reported in material and methods and classified as filamentous, fragmented or giant relative according to the mitochondrial morphology. ***(p < 0.001) denotes significance relative to CHIM3 strain using a 2 × 2 χ² test comparing filamentous vs fragmented + giant mitochondria. (F) Phenotypic analysis of diploid strains. Serial cell dilutions (5 × 10⁴, 5 × 10³, 5 × 10² and 5 × 10¹ cell/spot) of DW1.1 strain containing two copies of CHIM3 wild type (homoallelic diploid), a copy of CHIM3 wild type plus an empty plasmid (hemiallelic strain) or a CHIM3 wild type and a mutant chim3 copy (heteroallelic diploid) were spotted on YP medium, supplemented with either 2% glucose or 1.5% glycerol-1.5% ethanol. Pictures were taken after a 5-days incubation at 28 °C. A representative experiment out of three independent clones is shown. (G) Respiratory activity of homo-, hemi and heteroallelic strains. Values are means ± SD of three to four independent experiments and have been normalized to the respiratory activity of the homoallelic diploid. *(p < 0.05) and ***(p < 0.01) using Dunnett’s multiple comparison test. (H) Mitochondrial morphotypes percentage in diploid strains. 280 to 551 cells were classified as filamentous, fragmented or giant according to the mitochondrial morphology. ***(p < 0.001) denotes significance relative to homoallelic strain using a 2 × 2 χ² test comparing filamentous vs fragmented + giant mitochondria.
phenotype, we exploited the CHIM3 allele. Two of the four mutations studied in fibroblasts and MEF models (I382M and R445H) were previously described in our system [32], and here re-analyzed adding the further G439V, R445H and D603G mutations.

At first, the chim3 mutant alleles, obtained through mutagenic PCR, were introduced in the yeast centromeric (single copy) plasmid pFL39TETo, under the strong yeast promoter CYC1 fused with seven repeats of the tetO operator cassettes. Since deletion of MGM1 makes the strain irreversibly devoid of mtDNA, CHIM3 alleles were introduced in the haploid W303–1B mgm1 strain transformed with the plasmid pFL38MGM1, which was subsequently lost by the plasmid shuffling technique in order to obtain a strain expressing only the CHIM3 wt.

Concerning the haploid strains, we found that: (i) in all mutants, the oxidative growth was absent (Fig. 8A); (ii) no mtDNA was present, as measured with qPCR; (iii) the ratio of the l- and s-forms was unbalanced, although at different extent, except for strain harboring Q785R and V910D mutations. The respiratory activity of heteroallelic strains carrying the mutations was reduced for all strains except for that bearing I382M mutation (Fig. 7G), similar to the results of growth analysis. Finally, strains harboring G439V, R445H and D603G showed a significant decrease of cells with filamentous mitochondrial morphology, being again the strains R445H and D603G more affected (Fig. 7H). The results are summarized in Table 4.

### 3.4. Phenotypic profile of yeast strain harboring additional mutations in OPA1

Thanks to the flexibility of yeast in studying mutations in OPA1, here we have also investigated other seven substitutions found in patients with both DOA or DOA “plus” phenotype and localized in the different OPA1 domains (see positions within the OPA1 protein and other details in Table 1).

Concerning the haploid strains, we found that: (i) in all mutants, the oxidative growth was absent (Fig. 8A); (ii) no mtDNA was present, as measured with qPCR; (iii) the ratio of the l- and s-forms was unbalanced, although at different extent, except for strain harboring Q785R and V910D mutation (Fig. 8B and C); (iv) no filamentous mitochondrial morphology was observed (Fig. 8D).

Regarding the diploid strains, we found that most of the heteroallelic strains displayed a decreased oxidative growth, which was almost absent for strain harboring L593P mutation (Fig. 8E). On the contrary, in heteroallelic strains containing the Q785R and V910D mutations the growth was similar to that of the homallelic one. The respiratory activity (Fig. 8F) and the mitochondrial morphology (Fig. 8G) were grossly congruent with the oxidative growth phenotype, suggesting that all the mutations, except the Q785R and the V910D ones, behaved as dominant negative.

To ascertain whether these results were not influenced by the use of a chimeric gene, the S298N and I382M mutations, which involve conserved residues, were introduced at the corresponding position S241 N and I322 M in yeast Mgm1. The mgm1 haploid strain carrying
Fig. 8. Characterization of additional OPA1 mutations in yeast. (A) Phenotypic analysis of haploid strains performed as in Fig. 7A. (B) Western Blot analysis of CHIM3 expression level as in Fig. 7B. A representative experiment out of two is shown. (C) Ratio between the l−/s-forms normalized to the ratio observed in CHIM3. Data are means ± SD. (D) Mitochondrial morphotypes percentage in haploid strains measured as in Fig. 7E, analyzing 143 to 328 cells for each strain (E) Phenotypic analysis of diploid strains carried out as in Fig. 7F. (F) Respiratory activity of homo-, hemi and heteroallelic strains determined, as in Fig. 7G, on four to five independent clones. *p < 0.05, **p < 0.01 and ***p < 0.001 using Dunnett's multiple comparison test. (G) Mitochondrial morphotypes percentage in diploid strains as detailed in Fig. 7H, analyzing 288 to 737 cells for each strain. Measurement for the heteroallelic L593P could not be carried out due to the low growth level of this strain transformed with the mGFP vector on SC medium.

the S241 N mutation was unable to grow on respiratory carbon sources (data not shown), as observed for the chimeric strain with the equivalent mutation (Fig. 3A), but not for the I322M mutation, as previously reported [32]. In heteroallelic condition, the mgm1I322M allele behaved as dominant negative, with decreased oxidative growth and respiratory activity compared to both homoallelic (MGMI/MGM1) and hemiallelic (MGMI/empty plasmid) strains. On the contrary, heteroallelic strain harboring mgm1I322M, corresponding to the human mutation I328M, behaved as the homoallelic strain (Fig. S3A–B).

3.5. OPA1 structural model and mutations mapping

The structural determinants associated with the OPA1 mutations were analyzed through the calculations of structural models of OPA1. A homology model of human OPA1 was previously inferred on the basis of a distant homologue from the bacteria Nostoc punctiforme [11]. The recent release in the Protein Data Bank (PDB) of the structures of several OPA1 eukaryotic homologues, prompted us to develop a new OPA1 model. In particular, the new crystal structures feature the helical domain in a completely different conformation with respect to the GTPase domain, resulting in an elongated conformation (“open” conformation hereafter). We, thus, speculate that the bacterial OPA1 homologue from N. punctiforme structure shows the helical domain in a “close” conformation. Fig. 9A reports the homology models obtained for human OPA1 in the “open” and in the “close” conformations.

The OPA1 mutations were mapped on the new structural models (Fig. 9B). The I382M and G439 V are located near the G1 and G3 loops involved in the GTP binding and/or hydrolysis, conversely the R445H is positioned on the surface of the GTPase domain, in a region coming in contact with the helical domain in the “close” conformation, where it might impair the “closing” movement. The novel D603H sits in the “hinge” between the GTPase and the helical domains. An analogous structural mapping was performed for the other mutations analyzed in MEFs and in the yeast MGM1/OPA1 chimera: the R290Q, located in the structural mapping was performed for the other mutations analyzed in models generated ad hoc to evaluate cellular dysfunctions strictly dependent on OPA1 mutations: MEFs and yeasts. These models display a clear-cut phenotype that provides, by complementary information, a quick definition of mutation’s pathogenicity, thus proving useful for patient’s diagnosis. Strikingly, some mutant alleles literally behave as null mutations (G439 V, R445H and R290Q), when unopposed by the co-presence of the wt allele in MEFs, whereas at the opposite end a hypomorphic mutation such as I382M reveals only a slight damaging potential.

4.1. Fibroblast model

Fibroblasts from DOA patients are the commonest cell model used to date to analyze the effect of different OPA1 mutations, but suffer numerous limitations, such as great heterogeneity of phenotypes due to the individual nuclear and mitochondrial genomes variability and the presence of the wt allele. Furthermore, fibroblasts fail to display the features typically seen only in post-mitotic tissues, relevantly the mtDNA alterations (deletions/deletions). On the other hand, the experimental use of muscle tissue derived from patient’s biopsies is limited by the invasiveness of the procedure and the small amounts of material. Different degrees of energetic dysfunction, mitochondrial morphology and cristae alteration have been reported in DOA fibroblasts [11,21,23,24,30,46], particularly after enhancing the phenotype by metabolic stress conditions (DMEM-galactose) [24,30]. Furthermore, generation of primary fibroblast cultures takes a relatively long time and these cells suffer the disadvantage of a finite life span, undergoing replicative senescence.

In this study the mtDNA content, energetics and network morphology were assessed in fibroblasts bearing three well know OPA1 mutations (I382M, G439 V and R445H) and one new mutation (D603H). Remarkably, the G439 V and R445H mutations, associated with a similarly severe clinical phenotype, induced a substantial and opposite alteration in the mtDNA amount, a previously unrecognized feature in fibroblasts. The analysis of patient’s muscle bearing the G439 V mutation confirmed our previously reported result of an mtDNA content similar to controls [11]. Interestingly, for the R445H mutation, only the fibroblasts and corresponding muscle biopsy from patient II presented mtDNA depletion, whereas a normal amount of mtDNA was observed for patient I, notwithstanding the same reduction in OPA1 level in both cases. This observation suggests that other modifying factors might be influential. For example, considering the father-daughter segregation of the R445H mutation, the mtDNA haplogroup differs, as well as other unknown nuclear variants, the age and gender. Considering the limited number of patients explored in this study, an appropriate large-scale investigation on this issue is warranted. From the energetic point of view, the mutations did not significantly influence the supercomplexes assembly, although a general and significant decrease in cellular ATP levels and respiration was detected after metabolic stress. In glucose, the basal and maximal mitochondrial respirations were slightly but not significantly reduced only with the G439 V mutation, however both respiratory ratios were unaffected. Thus, although some energetic impairment was observed only after metabolic stress, a specific correlation with the mutation type could not be clearly identified. The only parameter that almost linearly correlated with the severity of patients’ clinical phenotype was the mitochondrial morphology in DMEM-galactose. As summarized in Table 2, the overall evaluation of the five readouts shows that each mutation presents one, two or three dysfunctional parameters, not linearly correlated with the patient’s clinical conditions.

4.2. MEFs model

As a second model, the MEF lines stably expressing isoform 1 have...
A

B
Mutated residues are reported with light blue spheres for the four mutations studied in fibroblasts and MEFs and green spheres for the mutations analyzed also in yeast and MEFs. 

Proved also extremely useful, since the quick and easy insertion of the OPA1 missense mutations allowed for a detailed biochemical investigation of the dysfunctional phenotypes, which were enhanced as compared to fibroblasts and nicely correlated with the clinical severity observed in the patients. In fact, both G439V and R445H mutations induced drastic mtDNA depletion, impaired respiration, remarkable supercomplexes disorganization and complete mitochondrial network fragmentation, which are all in excellent agreement with the clinical severity of these DOA “plus” patients. The same phenotypes were observed also for the R290Q and the G300E mutations [36], both associated with DOA “plus”. Opposite, the I382M mutation did not exhibit any significant dysfunction, except for its tendency to reduced respiration, in agreement with its role of mildly pathogenic or hypomorphic mutation. Interestingly, the novel D603H mutation, despite maintaining a normal mtDNA and ATP content, showed a slightly reduced respiration, a mild CV disassembly and a significant increase in mitochondrial network fragmentation, again in agreement with the intermediate clinical phenotype of these patients with pure DOA and severe visual dysfunction. Thus, as summarized in Table 3, in MEFs model the number of defective hallmarks linearly increased with the severity of clinical phenotype of the mutation, being only one affected for the I382M mutation, three for the D603H mutation and five for the G439V and R445H mutations. While the Q785R mutation presented normal mtDNA and partially affected mitochondrial morphology, similar to the D603H mutation, the S545R mutation had an intermediate behavior, borderline across phenotypes. Indeed, the complete fragmentation of the mitochondrial network was associated with only a slight, non-significant reduction of mtDNA amount, supporting a borderline nature of this variant, causing either DOA or DOA “plus” possibly depending on modulation of other genetic and/or environmental modifiers. MEFs analysis suggests that a complete fragmentation of the mitochondrial network, in association with mtDNA depletion, hallmarks the DOA “plus” phenotype, whereas if these two parameters are mildly affected the final outcome is expected to be pure DOA. This hypothesis is represented in the scheme of Fig. 10.

4.3. Yeast model

Switching to the yeast model, by using a chimeric gene, the greatest advantage was the insight on the genetic mechanism of OPA1 mutations and the kind of dominance of the mutations. In fact, CHIM3 homoallelic and hemiallelic strains showed no difference in the respiratory phenotype, contrary to humans, likely due to a stronger promoter of the CHIM3 allele, which guarantees a sufficient, but not toxic, expression in the diploid strain too. However, the comparison of the haploid and diploid models may discern three types of mutant alleles. (i) If the haploid strain shows a partially defective respiratory phenotype, i.e. a reduced oxidative growth and/or a reduced respiratory activity, and the heteroallelic strain behaves as the homo- and hemiallelic ones, then the mutant allele can be considered hypomorphic, as observed for I382M mutation. (ii) If the haploid strain displays no oxidative phenotype, i.e. is unable to grow on oxidative carbon sources, and the heteroallelic strain behaves as the hemiallelic one, then the mutant allele can be considered null, since it does not interfere with the activity of the wt allele. (iii) If the haploid strain displays no oxidative phenotype and the heteroallelic strain shows a decreased oxidative growth compared to the hemiallelic, the mutant allele can be considered antimorphic, resulting in a dominant negative phenotype.

The current and our previous study [32] clearly revealed that all mutations associated with DOA or DOA “plus” phenotype cause, in haploid strain, the inability to grow on oxidative carbon sources due to loss of mtDNA. The only exception is the I382M, which is either associated with a very mild phenotype [47] or it has been proposed to act as a hypomorphic allele [16,19].
While human OPA1 nonsense mutations are associated in most cases with non-syndromic DOA due to haploinsufficiency as a genetic mechanism, missense mutations can be associated with both pure DOA or DOA “plus”, due to either haploinsufficiency or negative dominance, depending on the mutation [13,48]. Yeast analysis revealed that most mutations, with the exception of two, behave as partially dominant due to a negative dominance. The reduced oxidative growth and respiratory activity of heteroallelic strain suggest that the mutant isoform interferes with the wt protein. This result suggests two scenarios: (i) most of the missense mutations found in humans are per se partially dominant negative, and the severity of the phenotype as defined by pure DOA or DOA “plus”, depends on the degree of interference of the mutant protein on wt in affecting mtDNA maintenance and mitochondrial fragmentation. In addition, some missense mutations may adjunctively impinge on the stability of the mutant protein itself. (ii) Alternatively, most mutations are dominant and have the potential to cause a DOA “plus” phenotype, and the severity of disease depends on other factors, such as the co-presence of modifying variants in other genes, as well as environmental factors or age/gender of the patients. This is the case for the S545R mutation, which was found associated with either DOA or DOA “plus” in different patients [13,31,45].

Intriguingly, we noticed that most mutations, including all the dominant negative ones, altered the relative amount of the l- and s-forms in the mutant haploid yeast strains compared with the wt, suggesting that such mutations can also impair the protein processing. In all cases, the levels of the s-form are decreased and, thus, the ratio of l- and s-forms is increased. By using both Opa1 null MEFs expressing different combination of OPA1 isoforms and OPA1 silenced HeLa cells, we have previously demonstrated that increased l- to s-forms ratio is associated with a highly fragmented mitochondrial network [36]. A similar phenotype was previously reported in yeast, where overexpression of the Mgm1 l-form, increasing the l- to s-forms ratio, lead to both loss of mtDNA and fragmentation of mitochondrial network [49]. Thus, increased l- to s-forms ratio in yeast could be used as an additional indicator of pathogenicity of the mutations.

4.4. Models comparison

The comparison of fibroblast and MEF results raises two observations deserving some further comments. First, there is a remarkable discrepancy in terms of OPA1 protein abundance, which is partially reduced in fibroblasts carrying the I382M, R445H and D603H mutations, whereas this is not affected in MEFs expressing the single mutant allele on isoform 1. The reduced amount of OPA1 protein was already noticed in other studies [15,50]. We envisage a possible explanation by hypothesizing that the interaction between mutant and wt protein may perturb OPA1 oligomers stability, favoring degradation. Second, we noticed that in patient’s derived fibroblasts CV and Ct-containing supercomplexes were properly assembled, at difference with MEFs carrying the most severe mutant mutations. We also determined a normal supramolecular organization in fibroblasts carrying OPA1 mutation causing haploinsufficiency mutations (Del Dotto and Zanna, unpublished results). These observations challenge the previously proposed relevance of supercomplex assembly as driving mechanism for the bioenergetics deficiency in DOA [51]. Considering that MEFs expressing the most severe OPA1 missense mutations displayed a significant perturbation of supercomplexes organization, there must be a sufficient complementation by the wt allele overcoming this potentially harmful mechanism in patient’s fibroblasts. This conundrum deserves further investigations.

In yeast model most OPA1 mutations strongly compromise mtDNA content, mitochondrial morphology, oxidative growth and energetics, making difficult to identify the degrees of severity related to the different mutations. However, thanks to the chimeric gene, yeast modeling is excellent to determining whether a mutation is pathogenic or not and to evaluating its dominance/recessivity. Conversely, the MEFs model nicely defines the biochemical features, highlighting also different degrees of severity associated with the mutations, but does not inform on dominance/recessivity. These two models are therefore complementary, operating in parallel to uncover DOA pathophysiology.

A further comparison of MEFs and haploid yeast strains showed that: (i) in both models, the mutant alleles bearing G439 V and R445H mutations behaved as a null allele concerning mtDNA content, mitochondrial morphology and respiratory activity; (ii) MEF with I382M showed only a slightly altered phenotype, whereas yeast showed a slight but statistically significant alterations of mtDNA levels and mitochondrial morphology; (iii) for the other mutations, which behaved as null in the yeast model, there was a partial overlap with the phenotype found in MEFs, since the S545R mutation caused a complete mitochondrial fragmentation associated with a slight decrease of mtDNA levels, whereas D603H and Q785R mutations caused substantial increase in cells with fragmented network and, for the former mutation, a decrease of the OCR/ECAR ratio.

Finally, the accurate mapping of all OPA1 mutations in the new OPA1 structural model showed that all mutations localized in the GTPase domain and involved in GTP binding/hydrolysis or GDP release may cause DOA or DOA “plus”. Interestingly, the mutations L593P, D603H and V910D, which appear harmless in the “open” conformation, reveal their detrimental potential only when considered in the “close” conformation, being located in the hinge of the two domains, thus interfering with the helical domain motion.

5. Conclusion

In conclusion, our current results strongly support the combined analysis of human, MEF and yeast cell models for “deep phenotyping” of OPA1 mutations and comprehensive dissection of their mechanistic effects. These cell models hold great promises to developing new therapeutic interventions, taking advantage of the yeast model for powerful high throughput screening of molecules, whose efficiency could be then further validated in the mouse and human cell models.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Conflicts of interest statement

The authors declare no financial conflict of interest that might be construed to influence the results or interpretation of the manuscript.

Authors contributions

Conceptualization, VDD, EB and CZ; Methodology, VDD, EB and CZ; Investigation, VDD, MF, FM, AM, SJA, LC, CLM, CN, EB and CZ; Resources, DC; Writing – Original Draft, VDD, EB and CZ; Writing – Review & Editing, VDD, TL, PG, DC, VC, MR, EB and CZ; Visualization, VDD, EB and CZ; Funding Acquisition, DC and CZ; Supervision, MR, TL, PG, EB and CZ; Project Administration, EB and CZ.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://

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