



Dominant optic atrophy: Culprit mitochondria in the optic nerve

Guy Lenaers, Albert Neutzner, Yannick Le Dantec, Christoph Jüschke, Ting Xiao, Sarah Decembrini, Sebastian Swirski, Sinja Kieninger, Cavit Agca, Ungsoo S Kim, et al.

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Dominant Optic Atrophy: culprit mitochondria in the optic nerve

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Dominant Optic Atrophy: culprit mitochondria in the optic nerve

Abstract,

Dominant optic atrophy (DOA) is an inherited mitochondrial disease leading to specific degeneration of retinal ganglion cells (RGCs), thus compromising transmission of visual information from the retina to the brain. Usually, DOA starts during childhood and evolves to poor vision or legal blindness, affecting the central while sparing the peripheral visual field. In 20 % of cases, DOA presents as syndromic disorder, with secondary symptoms essentially affecting neuronal and muscular functions. Twenty years ago, we evidenced that heterozygous mutations in *OPA1* are the most frequent molecular cause of DOA. Since then, variants in additional genes, which functions in many instances converge to *OPA1*'s functions, were identified by next generation sequencing. *OPA1* encodes a dynamin-related GTPase imported into mitochondria and located to inner membrane and intermembrane space. The many *OPA1* isoforms, resulting from alternative splicing of three exons, form complex homopolymers that on one hand give structure to mitochondrial cristae, and on the other contribute to fusion of the outer membrane, thus shaping the whole mitochondrial network. Moreover, *OPA1* is required for oxidative phosphorylation, maintenance of mitochondrial genome, calcium homeostasis and regulation of apoptosis, thus making *OPA1* as the *Swiss army-knife* of mitochondria. Understanding DOA pathophysiology necessitates to identify peculiarities of RGC function with respect to *OPA1*. Besides the tremendous energy requirements of RGCs to relay visual information from the eye to the brain, these neurons present unique features related to their differential environments in the retina, and after the lamina cribrosa, in the optic nerve, which parallel major adaptations of mitochondrial physiology and shape. Three DOA mouse models, with different *Opal* mutations, have been generated to study intrinsic mechanisms responsible for RGCs degeneration, while they further disclosed secondary symptoms related to mitochondrial dysfunctions, mirroring the syndromic DOA patient presentations. Metabolomic analyses of cells, mouse organs and patient plasma mutated for *OPA1* revealed new unexpected pathophysiological mechanisms related to mitochondrial dysfunctions, correlating quantitatively to the severity of the disease. We here review

and synthesize all these data, and propose different approaches for embracing possible therapies to fulfil unmet medical needs of this disease and the hope of DOA patients.

Keywords:

Dominant Optic Atrophy; optic nerve; retinal ganglion cells; mitochondria; OPA1;

List of abbreviations:

AAV: adeno-associated viral vector;

AON: antisense oligonucleotide;

ATF: artificial transcription factor;

bp: base pair;

BBSOAS: Bosch-Boonstra-Schaaf optic atrophy syndrome;

CMT2A: Charcot-Marie-Tooth neuropathy type 2A

COX: cytochrome oxydase;

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

Cas9: CRISPR associated protein 9

dCas9: dead Cas9;

DOA: dominant optic atrophy;

DOAC: dominant optic atrophy plus cataract;

DOAplus: syndromic dominant optic atrophy;

DRP1: Dynamin-related Protein 1

ER: endoplasmic reticulum;

ERG: electroretinogram;

GED: GTPase effector domain;

IMM: inner mitochondrial membrane;

IMS: intermembrane space

IOP: intraocular pressure;

ipRGC: intrinsically photosensitive retinal ganglion cells;

LC: lamina cribrosa;

LHON: Leber's hereditary optic neuropathy;

LogMAR: Logarithm of the Minimum Angle of Resolution

l-OPA1: long OPA1 isoform;

MAM: ER-mitochondrial associated membranes;

MFN2: Mitofusine 2

MS: :multiple sclerosis;

mtDNA: mitochondrial DNA;

MTS: mitochondrial targeting sequence;

NGS: next generation sequencing;

OCT: optical coherence tomography;
OMA1: mitochondrial peptidase
OMM: outer mitochondrial membrane;
ON optic nerve;
OPA1: optic atrophy 1;
OXPHOS: oxidative phosphorylation;
RGC: retinal ganglion cells;
RNFL: retinal nerve fibre layer;
SHIRPA: SmithKline Beecham, Harwell, Imperial College, Royal London Hospital, phenotype assessment;
s-OPA1: small OPA1 isoform;
TALE: transcriptor activation like effectors;
U1: small nuclear ribonucleoprotein U1;
uORF: upstream open reading frame;
UTR: untranslated region;
VEP: visual evoked potential;
YME1L : i-AAA protease
ZFP: zinc finger protein.

List of contents

KEYWORDS.....	1
LIST OF ABBREVIATIONS.....	1
ABSTRACT	5
MAIN BODY OF MANUSCRIPT	6
1. DOA IN CLINICS	6
1.1. Common clinical features of DOA.....	6
1.2. Expanding DOA genotypes and phenotypes.....	7
2. GENETICS AND RISK FACTORS.....	9
2.1. Mutations in OPA1	9
2.2. OPA1 Phenotype-genotype correlations	10
2.3. Additional DOA loci and genes	10
2.4. Genetic diagnostics	13
3. THE CULPRIT MITOCHONDRIA	14
3.1. OPA1 structure and expression	14
3.2. OPA1: a mitochondrial “Swiss army-knife”	15
3.3. OPA1 and cristae structure and remodeling	16
3.4. OPA1 in mitochondrial fusion.....	16
3.5. OPA1 and the control of energy metabolism	18
3.6. OPA1 in the regulation of mitochondrial calcium uptake	18
3.7. OPA1 and the control of apoptosis.....	18
3.8. OPA1 and the maintenance of the mitochondrial genome	19
3.9. Alternative extra-mitochondrial OPA1 functions	20
3.10. Functions of the other genes involved in DOA.....	20
4. THE PATHOPHYSIOLOGY OF DOA	21
4.1. Why RGCs and the optic nerve degenerate in DOA?.....	21
4.2. Why specifically the RGCs from the papillo-macular bundle degenerate in DOA?.....	22
5. ANIMAL MODELS OF DOA	23
5.1. Mouse models of DOA.....	23
5.2. Characterization of the Opa1 ^{+/-del} mouse model of DOA.....	25
5.3. Expression of DOA phenotypes in the Opa1 ^{+/-STOP} mouse model.....	26
5.4. The Opa1 ^{+/-delTTAG} model of DOA.....	27
5.5. Modeling DOA in other organisms	28
6. METABOLOMICS CONSIDERATIONS IN DOA.....	29
6.1. Qualitative aspects.....	29
6.2. Quantitative aspects	30
7. CONCLUSIONS AND FUTURE DIRECTIONS TOWARDS THERAPIES	31
7.1. Gene therapy for DOA	31
7.2. Pharmacological and micro-nutritional protection of the optic nerve	35
7.3. The ultimate goal: RGC and optic nerve regeneration	37
LEGENDS TO THE FIGURES.....	38
Figure 1. Ophthalmological findings in a 26-year-old man with a confirmed pathogenic OPA1 mutation (c.1057A>C, p.Ser353Arg).	38
Figure 2. Genes causing DOA and their associated phenotypes.	38
Figure 3. Consequences of OPA1 dysfunction on the IMM and OMM shapes.	38

Figure 4. Assessment of mitochondrial abundance in mouse retinal ganglion cells and the optic nerve.....	38
Figure 5: Electron microscopy showing the changes in density and morphology of mitochondria along the optic nerve in relation to myelination.	38
Figure 6: Schematic representation of a retinal ganglion cell and its dual environmental conditions before and after the lamina cribrosa.....	39
Figure 7: Chronological comparison of the molecular and phenotype features associated with three <i>Opa1</i> mouse models.....	39
Figure 8: Synthetic metabolomic signatures associated with OPA1 dysfunction.....	39
DECLARATION OF INTERESTS.....	40
ACKNOWLEDGEMENTS	40
REFERENCES	41

Abstract

Dominant optic atrophy (DOA) is an inherited mitochondrial disease leading to specific degeneration of retinal ganglion cells (RGCs), thus compromising transmission of visual information from the retina to the brain. Usually, DOA starts during childhood and evolves to poor vision or legal blindness, affecting the central while sparing the peripheral visual field. In 20 % of cases, DOA presents as syndromic disorder, with secondary symptoms essentially affecting neuronal and muscular functions. Twenty years ago, we evidenced that heterozygous mutations in *OPA1* are the most frequent molecular cause of DOA. Since then, variants in additional genes, which functions in many instances converge to *OPA1*'s functions, were identified by next generation sequencing. *OPA1* encodes a dynamin-related GTPase imported into mitochondria and located to inner membrane and intermembrane space. The many *OPA1* isoforms, resulting from alternative splicing of three exons, form complex homopolymers that on one hand give structure to mitochondrial cristae, and on the other contribute to fusion of the outer membrane, thus shaping the whole mitochondrial network. Moreover, *OPA1* is required for oxidative phosphorylation, maintenance of mitochondrial genome, calcium homeostasis and regulation of apoptosis, thus making *OPA1* as the *Swiss army-knife* of mitochondria. Understanding DOA pathophysiology necessitates to identify peculiarities of RGC function with respect to *OPA1*. Besides the tremendous energy requirements of RGCs to relay visual information from the eye to the brain, these neurons present unique features related to their differential environments in the retina, and after the lamina cribrosa, in the optic nerve, which parallel major adaptations of mitochondrial physiology and shape. Three DOA mouse models, with different *Opal* mutations, have been generated to study intrinsic mechanisms responsible for RGCs degeneration, while they further disclosed secondary symptoms related to mitochondrial dysfunctions, mirroring the syndromic DOA patient presentations. Metabolomics analyses of cells, mouse organs and patient plasma mutated for *OPA1* revealed new unexpected pathophysiological mechanisms related to mitochondrial dysfunctions, correlating quantitatively to the severity of the disease. We here review and synthesize all these data, and propose different approaches for embracing possible therapies to fulfil unmet medical needs of this disease and the hope of DOA patients.

Main body of manuscript

1. DOA in Clinics

1.1. Common clinical features of DOA

Describing several large families with unequivocally autosomal dominant inheritance, Poul Kjer in Denmark and Wolfgang Jäger in Germany (Jaeger, 1954; Kjer, 1957) were the first ophthalmologists to establish a dominant form of isolated optic atrophy, genetically and clinically distinct from the Leber Hereditary Optic Neuropathy (LHON, MIM#535000) (Chun and Rizzo, 2017). In their initial description of dominant optic atrophy (DOA, MIM#165500), it was characterized by progressive bilateral visual loss and color vision defects, related to optic nerve degeneration starting in early childhood (Jaeger, 1954; Kjer, 1957). The onset of symptoms in DOA probably starts during the first decade of life, but usually the diagnosis occurs several years later during the second decade of life, because of the difficulty in appreciating the visual loss by the young patients or their care-takers (Lenaers et al., 2012; Votruba et al., 1998). Typically, bilateral presentation is the norm, but it may start asymmetrically as well. There can be marked variation in DOA severity with visual acuity ranging from 20/20 to light perception, with around 40% of patients having a visual acuity over 20/60 (Pineles et al., 2019). In one longitudinal case series, the mean visual acuity was 0.94 LogMAR (20/173) and the mean rate of visual loss was 0.032 LogMAR/year, which approximates to three lines of vision per decade of life (Yu-Wai-Man et al., 2010a). Fundus examination will reveal a stereotypic white or grey wedge-like zone mainly on the temporal portion of the optic disc, but sometimes affecting all quadrants. Visual field perimetry typically shows a bilateral central or caeco-central scotoma consistent with preferential involvement of the papillomacular bundle, while the peripheral visual field remains normal in most cases. Optical coherence tomography (OCT) imaging of the optic nerve head demonstrates a reduction of the retinal nerve fiber layer (RNFL) thickness, witnessing the prominent loss of retinal ganglion cells (RGCs) in the temporal quadrant, especially in the area of the papillomacular bundle corresponding to the central visual field defects (Figure 1) (Asanad et al., 2019). Macular OCT examination disclose a thinning of the ganglion cell layer (Corajevic et al., 2018), and angiographic OCT imaging will further show reduced blood flow in the temporal region of the optic disc (Balducci et al., 2017; Himori et al., 2017; Inoue et al., 2016). Color vision loss reflects a generalized dyschromatopsia, sometimes occurring specifically in the blue-yellow spectrum, contrasting with the red-green dyschromatopsia observed in LHON (Votruba et al., 1998). The pupillary reflex and circadian rhythms are not affected in DOA, as the intrinsically photosensitive RGCs seem spared during the course of the disease. Notably, the optic nerve head in DOA patients has been reported to be significantly smaller compared with healthy controls (Barboni et al., 2010). It should be stressed that even within the same family, the former features may be presented with marked differences in the severity and in the rate of disease progression (Hayashi et al., 2017). DOA is genetically heterogeneous with an increasing number of reported genetic loci and disease-causing genes recently identified through the introduction of next generation sequencing

technologies (Jurkute et al., 2019b; Lenaers et al., 2012). Nevertheless, about 70% of patients with DOA harbor pathogenic mutations in OPA1 (3q28-q29), the only gene which was primarily identified as causing DOA (Alexander et al., 2000; Delettre et al., 2000). All other DOA-associated genes were first identified as responsible for other syndromes, and only later shown to cause also nonsyndromic DOA. OPA1 encodes for a dynamin-like GTPase protein located within the inner mitochondrial space associated to the inner membrane (IMM) (Olichon et al., 2002). Therefore, DOA is basically a mitochondrial disease of nuclear origin, while the other most common form of inherited optic neuropathy, LHON, is caused by variants in the mitochondrial genome. OPA1 variants are mostly stably inherited within families although family history may be not always informative due to sub-clinical disease expression and reduced penetrance. Only in few instances de novo OPA1 variants have been reported (Baris et al., 2003; Bolognini et al., 2017; Cohen et al., 2016; Zerem et al., 2019). As many patients with DOA will evolve to legal blindness, there is an urgent area of unmet clinical need.

1.2. Expanding DOA genotypes and phenotypes

Although optic atrophy is the defining feature of all OPA1-related DOA individuals, multisystem manifestations are reported in up to 20% of OPA1 mutation carriers (Amati-Bonneau et al., 2003; Amati-Bonneau et al., 2008; Hudson et al., 2008; Payne et al., 2004; Yu-Wai-Man et al., 2010b). The clinical features reported in the syndromic forms called DOAplus include myopathy, peripheral neuropathy, ataxia, encephalopathy, sensorineural hearing loss, and chronic progressive external ophthalmoplegia (Van Bergen et al., 2011). There is a significant difference in the frequency of mutation subtypes between patients with pure optic atrophy and those with DOAplus phenotypes. Indeed, missense OPA1 mutations located within the GTPase catalytic domain are more likely to cause a more severe syndromic phenotype compared with mutations resulting in haploinsufficiency, such as deletions and splice site mutations (Ham et al., 2019; Yu-Wai-Man et al., 2011b). The more severe impact of mutations on OPA1 function can be associated with an increased loss of RGCs. This also accounts for faster rates of visual decline and the worse visual outcome in patients with DOAplus compared to DOA patients (Yu-Wai-Man et al., 2011a; Yu-Wai-Man et al., 2010b).

More recently, bi-allelic OPA1 mutations were associated with patients clinically presenting with Behr syndrome, a distinct severe neurological syndromic disease featuring early-onset optic atrophy, spasticity, spinocerebellar ataxia, peripheral neuropathy, gastrointestinal dysmobility and intellectual disability. Notably, most of these cases are compound heterozygous with the hypomorphic variant c.1311A>G/p.Ile437Met, referring to NM_130837.3, in trans with a loss of function variant (Bonifert et al., 2014; Bonneau et al., 2014; Carelli et al., 2015a; Carelli et al., 2015b; Nasca et al., 2017; Schaaf et al., 2011). Additionally, rare associations of heterozygous or bi-allelic OPA1 mutations have been reported in patients presenting with optic atrophy and spastic paraparesis (Yu-Wai-Man et al., 2010b), multiple sclerosis-like syndrome (Verny et al., 2008), syndromic parkinsonism and dementia (Carelli et al., 2015a; Lynch et al., 2017), severe syndromic cardiomyopathy associated to myopathy and

encephalopathy (Spiegel et al., 2016), and Behr-like syndrome with metabolic stroke (Zerem et al., 2019), highlighting the complexity of OPA1-linked phenotypes.

The complexity to infer clear genotype–phenotype correlations in OPA1-associated diseases is further supported by the highly variable clinical severity both between and within families carrying the same mutation (Thiselton et al., 2002). This implies that yet undefined genetic, epigenetic and environmental factors are likely to modulate the phenotypic expression of DOA. Although challenging, untangling these modifiers will be key to our understanding of the pathological pathways contributing to the preferential loss of RGCs in DOA and the variable tissue specificity encountered in the DOAplus forms of the disease (Yu-Wai-Man et al., 2016). Several contributing factors leading to the development of DOAplus features have been put forward. First, only missense OPA1 mutations were identified in such clinical presentations, with a peculiar genotype-phenotype correlation related to the R510H OPA1 mutation, which systematically leads to optic atrophy and neuro-sensorial deafness (Amati-Bonneau et al., 2003; Chen et al., 2007; Lodi et al., 2004). Second, as the catalytic GTPase domain of OPA1 protein plays a critical role in mitochondrial homeostasis, it is not surprising that missense mutations in this essential domain increase the risk of DOAplus potentially due to a dominant-negative effect of the mutation (Yu-Wai-Man et al., 2011b). This pathomechanism was suggested to result in partially inactive OPA1 homopolymers (Olichon et al., 2006). Intriguing findings concerning DOAplus are the presence of mitochondrial DNA (mtDNA) deletions in skeletal muscle biopsies in combination with cytochrome c oxidase (COX)-deficient muscle fibres (Amati-Bonneau et al., 2008; Hudson et al., 2008). A high proportion of COX-deficient muscle fibres is pathognomic of mitochondrial diseases. It was proposed that the accumulation of mtDNA deletions is secondary to post-mitotic clonal expansion, facilitated by the mitochondrial fragmentation induced by OPA1 mutations (Payne et al., 2013; Yu-Wai-Man et al., 2010c). However, it is possible that mtDNA instability in OPA1 disease represent an epiphenomenon, in which the accumulation of mtDNA deletions does not contribute to the neuronal loss *per se* (Alavi et al., 2009).

Specific OPA1 polymorphic variants have also been associated with normal tension glaucoma, but not high tension glaucoma (Kirkman et al., 2009; Yu-Wai-Man et al., 2010d). Although further investigations are needed to clarify whether and how OPA1 variants could influence the development of glaucoma, it seems reasonable to have a lower intraocular pressure (IOP) threshold to treat a patient with DOA with consistently elevated IOP and an already compromised optic nerve. This is further supported by the fact that elevated hydrostatic pressure in an in vitro cell model triggers detrimental mitochondrial changes similar to that of OPA1^{+/-} cells, including mitochondrial fission, abnormal cristae and release of pro-apoptotic molecules (Ju et al., 2009).

Although smoking is an established risk factor for visual loss in LHON, the influence of smoking on disease progression in DOA has not been evaluated due to the practical difficulties involved in conducting such a study (Guo et al., 2012).

2. Genetics and risk factors

2.1. Mutations in OPA1

A first locus for DOA was mapped to chromosome 3q28-q29 by means of linkage analysis (Bonneau et al., 1995; Eiberg et al., 1994; Lunkes et al., 1995; Votruba et al., 1998; Votruba et al., 1997) and subsequently the disease-causing gene, termed OPA1 (for optic atrophy 1), was identified in parallel by positional cloning (Alexander et al., 2000) and as the human orthologue of the fission yeast Msp1 gene (Delettre et al., 2000). Initially, OPA1 was described with 30 coding exons (exons 1-30) and one non-coding exon (exon 31), among which exons 4, 4b and 5b were alternatively spliced resulting in eight coding mRNA transcripts (Delettre et al., 2001). Recently, two additional transcripts (9: NM_001354663, and 10: NM_001354664) containing an extra non-coding exon have been annotated, generating an N-terminally shortened protein starting within exon 3.

OPA1 is the major gene mutated in DOA, being responsible for about 70 % of cases in large cohorts (Almind et al., 2012; Ferre et al., 2009). More than 400 distinct likely pathogenic variants in OPA1 have been reported so far (Human Gene Mutation Database [HGMD]; Leiden Open Variant Database on OPA1 variants at <https://databases.lovd.nl/shared/genes/OPA1>; (Le Roux et al., 2019). Only few of these variants have been recurrently reported, such as the c.2873_2876del/ p.Val958Glyfs*3, the c.2635C>T/p.Arg879*, and the c.2991_3001delinsGGATGCTCCA/p.Arg998Aspfs*25, the latter commonly found in Danish DOA patients (Almind et al., 2012; Le Roux et al., 2019). Disease-causing variants are broadly scattered over the entire coding sequence, however, very few have been reported for the alternatively spliced exons (Cornille et al., 2008). Of the reported pathogenic OPA1 variants, 28% are missense variants; 24% are classified to induce aberrant splicing; 22% are frameshift variants; 15% are nonsense variations; and 7% are structural variants. Most of the missense variants cluster to the GTPase domain (exons 8-16) (Le Roux et al., 2019). Overall, the majority of disease-causing variants lead to premature termination of translation and thus likely results in a null allele. Consequently, reduced OPA1 levels are frequently observed in Western blots of patient-derived fibroblasts (Zanna et al., 2008). Hence, haploinsufficiency has been proposed as the predominant disease mechanism underlying OPA1-linked DOA (Le Roux et al., 2019; Pesch et al., 2001). Given the considerable fraction of disease-causing structural variants at the OPA1 gene locus (Almind et al., 2011; Fuhrmann et al., 2009), copy number variant analysis is recommended in routine genetic diagnostics. Moreover, deep intronic variants causing aberrant transcript splicing have been described recently (Bolognini et al., 2017; Bonifert et al., 2014) which may not be discovered by exon-centred genetic analysis. Yet, the frequency of such ‘hidden’ mutations most probably resulting again in null alleles, remains to be determined. A major proportion of DOAplus cases is associated with missense variants causing amino acid

substitutions in the GTPase domain of the protein, most probably leading to a dominant-negative effect of these DOAplus-linked variants (Amati-Bonneau et al., 2008; Chao de la Barca et al., 2020; Del Dotto et al., 2018b).

2.2. OPA1 Phenotype-genotype correlations

Genetic penetrance describes the probability that a specific genotype expresses the associated phenotype. Early reports noted a high penetrance in DOA families (Jaeger, 1954; Kivlin et al., 1983; Kjer, 1957). Subsequently, larger series of DOA patients and families linked to OPA1 mutations revealed that penetrance may vary considerably, between 43 to 88 % (Cohn et al., 2007; Fuhrmann et al., 2010; Toomes et al., 2001). This may be due in part to the clinical variability in among DOA families and even within families with some gene carriers being only subclinically affected (Votruba et al., 1998), thus hampering clear phenotype-genotype correlations in OPA1-linked DOA. Nonetheless, missense mutations tend to be associated with severe forms of DOA or DOAplus (Amati-Bonneau et al., 2008; Barboni et al., 2014; Hudson et al., 2008; Yu-Wai-Man et al., 2010b). The biochemical and mitochondrial phenotype of some of these missense variants have been studied intensively in patient-derived fibroblasts, murine MEFs and yeast, and showed good correlation with the clinical severity (Chao de la Barca et al., 2020; Del Dotto et al., 2018b; Lenaers et al., 2002). Similarly, patients with biallelic OPA1 mutations do frequently present with severe syndromic disease, such as the Behr- and Behr-like syndromes (Bonifert et al., 2014; Bonneau et al., 2014; Carelli et al., 2015b; Nasca et al., 2017; Schaaf et al., 2011; Yu-Wai-Man et al., 2010b). Nevertheless, a patient with isolated optic atrophy due to bi-allelic OPA1 mutations has also been described (Pesch et al., 2001). Notably, no patient with OPA1 bi-allelic loss of function variants has been described, as such genotype is most likely embryonic lethal, as suggested by experiments in mouse and drosophila (Alavi et al., 2007; Davies et al., 2007; Yarosh et al., 2008). Thus, at least one of the two variants observed in bi-allelic is considered a hypomorphic variant conferring some residual OPA1 function. A well-studied example is the c.1311A>G / p.Ile437Met variant which is *per se* not pathogenic even in a homozygous state (Bonifert et al., 2014) and results benign biochemical and mitochondrial phenotype (Chao de la Barca et al., 2020; Del Dotto et al., 2018b), but may act as a modifier associated with Behr syndrome if occurring in combination with a loss-of-function OPA1 variant in trans (Bonifert et al., 2014; Bonneau et al., 2014; Carelli et al., 2015b; Nasca et al., 2017; Schaaf et al., 2011).

2.3. Additional DOA loci and genes

Besides OPA1, mutations in a number of other genes were associated with DOA (Figure 2, Table 1). The majority of these genes encode proteins directly linked to mitochondrial function, mostly mitochondrial dynamics. All of them were primarily identified in the context of syndromic neurodegenerative diseases, and are only rarely associated with isolated DOA (Wissinger, 2018). It appears that mutations in OPA1 by default cause non-syndromic DOA, whereas syndromic forms of optic atrophy only occur in cases with dominant-negative or bi-allelic mutations. *Vice versa*, most other

DOA-associated genes were identified as causing syndromic diseases, often including optic atrophy, and subsequently associated with isolated optic atrophy (Figure 2 and Table 1).

The first example concerns WFS1, in which mutations have been primarily identified as causing the Wolfram syndrome, an autosomal recessive neurodegenerative disorder primarily associating optic atrophy and diabetes, then hearing loss, renal deficits and mental disease (Inoue et al., 1998). Wolframin, the protein product of WFS1, is an endoplasmic reticulum (ER) membrane glycoprotein that is enriched at mitochondria-associated ER membranes and plays a crucial role in intracellular calcium homeostasis (Delprat et al., 2018; La Morgia et al., 2020). WFS1 mutations have been described in several families with DOA and neuro-sensorial deafness (Chaussonnet et al., 2015; Grenier et al., 2016; Hogewind et al., 2010; Rendtorff et al., 2011), in Wolfram-like syndrome with variable expression of glucose intolerance (Eiberg et al., 2006; Valero et al., 2008), as a cause of autosomal dominant inherited deafness (Bespalova et al., 2001; Young et al., 2001), and in recessive isolated optic atrophy (Grenier et al., 2016).

A second example of DOA gene is OPA3. OPA3 encodes a protein located in the mitochondrial inner membrane, thought to play a role in the organization and shape of mitochondria, however, its exact function is still unknown (Da Cruz et al., 2003). Mutations in OPA3 were first identified to cause autosomal recessive 3-methylglutaconic aciduria type III or Costeff syndrome which is characterized by optic atrophy, spasticity and movement disorders (Anikster et al., 2001; Costeff et al., 1989). Later, single heterozygous missense mutations were identified in OPA3 in two multi-generation families affected by DOA and cataract (DOAC). Thus OPA3 may be associated with recessive as well as dominant optic atrophies (Reynier et al., 2004). Since then, several additional cases or families with dominant OPA3 mutations have been described, in which optic atrophy is frequently associated with extraocular features such as cataract, sensorineural hearing loss, peripheral neuropathy, ataxia and areflexia (Aygnac et al., 2012; Bourne et al., 2017; Grau et al., 2013; Horga et al., 2019; Li et al., 2017). As found in OPA1 and WFS1 patient cohorts, a high intra- and inter-familial clinical variability of OPA3-related dystrophies may occur, including cases of isolated optic atrophy (Sergouniotis et al., 2015).

Mutations in AFG3L2 encoding the ‘AFG3 like matrix peptidase subunit 2’, a m-AAA-protease essential for mitochondrial protein quality control, have been initially identified to cause spinocerebellar ataxia type 28 (SCA28) and spastic ataxia type 5 (Di Bella et al., 2010; Pierson et al., 2011). Subsequently, a specific missense variant, c.1402C>T/p.R468C, has been reported independently in individuals with DOA, associated with mild intellectual disability (Charif et al., 2015; Colavito et al., 2017). In two recent systematic studies, the screening of large series of OPA1-WFS1-OPA3 negative DOA patients identified diverse dominant and bi-allelic AFG3L2 variants in 20 families with isolated optic atrophy, or DOA associated with variable neurological symptoms (Caporali et al., 2020; Charif et al., 2020). Notably, all DOA-associated missense variants cluster in the ATPase domain of the protein,

which has never been found mutated in SCA28 patients. As no symptom of ataxia was associated to DOA related to AFG3L2 mutations and no optic atrophy has been reported for SCA28 patients, mutations in different domains of AFG3L2 are responsible for two neurological diseases without clinical overlap (Caporali et al., 2020; Charif et al., 2020).

Similarly, recessive mutations in SPG7, the orthologue of AFG3L2 gene, were identified in patients with hereditary spastic paraplegia type-7 (HSP7) (Casari et al., 1998). Subsequently, dominant SPG7 mutations were identified in a large multi-generation DOA family and in seven index cases with DOA (Charif et al., 2020; Klebe et al., 2012). Notably, all dominant SPG7 variants identified in DOA patients are interspersed with the recessive ones responsible for HSP7. As optic atrophy has been described now in almost all HSP7 patients (Klebe et al., 2012), there is a clear overlap between the spastic paraplegia and the optic atrophy symptoms in the HSP7 recessive cases, although no symptom of paraplegia was observed in the SPG7-related DOA patients (Charif et al., 2020).

‘Dynamin 1-like protein (DNM1L) also known as ‘Dynamin-related Protein 1’ (DRP1) is another dynamin-related GTPase associated with mitochondria. In contrast to OPA1, DRP1 primarily localizes to the cytoplasm and is recruited to the mitochondrial outer membrane as an oligomeric complex to drive the constriction and scission of both mitochondrial membranes during mitochondrial network fission (Liu and Chan, 2015). Mutations in DNM1L have been described initially to cause infantile encephalopathy (Fahrner et al., 2016; Waterham et al., 2007), and epileptic encephalopathy (Vanstone et al., 2016; Verrigni et al., 2019). Later on, single heterozygous DNM1L missense mutations were identified in three multi-generation families segregating mild DOA, inducing a hyper-filamentous mitochondrial network in patient-derived fibroblasts, the opposite to what is observed in cells bearing OPA1 mutation (Gerber et al., 2017).

Mitofusin-2 encoded by the MFN2 gene is another key player in mitochondrial fusion (Praefcke and McMahon, 2004). MFN2 is located anchored to the outer membrane (OMM) of the mitochondria and promotes fusion of the OMM, whereas OPA1 is responsible for the IMM fusion. Mutations in MFN2 are primarily linked to Charcot-Marie-Tooth neuropathy type 2A (CMT2A), the most common form of axonal CMT (Zuchner et al., 2004). Some patients with axonal CMT and optic atrophy are sub-classified as ‘Hereditary Motor and Sensory Neuropathy Type VI’ (HMSN VI), and were found to harbour MFN2 mutations (Guerriero et al., 2020; Leonardi et al., 2015; Rouzier et al., 2012; Zuchner et al., 2006). In addition, optic atrophy is now found in 12% of CMT2A patients with MFN2 mutation (Bombelli et al., 2014). Notably, the optic atrophy in MFN2-linked cases occurs with an early or late onset, can be subacute or acute, and a considerable fraction of patients re-improve vision, which is distinct from other forms of DOA, but reminiscent of the course of visual loss and recovery in some LHON cases (Leonardi et al., 2015; Zuchner et al., 2006).

Autosomal dominant inherited Bosch-Boonstra-Schaaf optic atrophy syndrome (BBSOAS) is a rare condition characterized by visual loss associated with optic atrophy, optic nerve hypoplasia or cortical visual impairment, developmental delay and intellectual disability, and is caused by mutations in the NR2F1 gene (Bosch et al., 2014). Additional clinical BBSOAS descriptions featured patients with hypotonia, oromotor dysfunction, morphological changes of brain structures including thinning of the corpus callosum, and autism spectrum disorder, in addition to the optic atrophy (Chen et al., 2016; Kaiwar et al., 2017; Rech et al., 2020). NR2F1 encodes an orphan nuclear receptor protein involved in transcriptional regulation. A large number of experimentally validated or predicted target genes have been described for NR2F1 including genes encoding mitochondrial proteins (Montemayor et al., 2010; Pereira et al., 2000). Missense variants located in the DNA binding domain of NR2F1 or full gene deletions predominate in BBSOAS, nearly all of them occurring de novo (Bosch et al., 2014; Chen et al., 2016; Kaiwar et al., 2017).

Recently, mutations in SSBP1 encoding the mitochondrial single-stranded DNA-binding protein, required for mtDNA replication, have been identified in multiple large DOA families where optic nerve degeneration is sometimes associated to retinopathy to the optic nerve degeneration (Del Dotto et al., 2020; Jurkute et al., 2019a; Piro-Megy et al., 2020). In few additional families, more complex phenotypes featuring renal disease and deafness were reported (Del Dotto et al., 2020). All mutations identified are missense substitutions localizing to the DNA binding domain of SSBP1, eventually impairing mtDNA replication and causing mtDNA depletion (Del Dotto et al., 2020; Piro-Megy et al., 2020).

Furthermore, three loci for DOA have been identified by means of linkage analysis: OPA4 on chromosome 18q12.2-q12.3 (Kerrison et al., 1999), OPA5 on chromosome 22q12.1-q13.1 (Barbet et al., 2005) and OPA8 on chromosome 16q21-q22 (Carelli et al., 2011). However, the two OPA5 families were later on re-investigated by exome sequencing and found to carry the DNMT1L mutations on chromosome 12p11.21, thus disproving the existence of the OPA5 locus on chromosome 22q (Gerber et al., 2017).

2.4. Genetic diagnostics

Genetic diagnostics is considered standard of care for patients clinically diagnosed with non-inflammatory bilateral optic atrophy. Molecular analysis may thus confirm the presence of a hereditary form of optic atrophy, specify the mode of inheritance and define the genetic risk for the progeny, and eventually direct medical care for the early detection of extra-ocular pathologies. Genetic diagnostics may also be used to identify and counsel healthy or sub-clinically affected gene carriers in DOA families who have a 50% risk to transmit the mutated gene to each of their children. Disease-causing variants in OPA1 are the major cause of DOA including a considerable fraction of patients with extra-ocular symptoms and, thus, OPA1 is the recommended choice for a targeted genetic analysis. Yet, the state-of-

the art molecular diagnostics nowadays uses targeted next generation sequencing (NGS) of gene panels or whole exome sequencing, which cover most or eventually all of the known DOA genes. However, depending on the platform and laboratory settings, structural genomic alterations such as heterozygous deletions and insertions may still be poorly detected by these targeted NGS approaches. Therefore, copy number variation analysis such as multiplex-ligation-dependent probe analysis (Fuhrmann et al., 2009) should be considered to complement targeted sequencing. Yet, all these approaches may fall short for the detection of deep-intronic mutations as described for OPA1 (Bonifert et al., 2014), mutations in regulatory sequences or larger inversions. The fraction of such hidden mutations still remains to be determined. Whole genome sequencing would be able to detect such aberrations, however, technical issues in data analysis and costs currently prohibit its application to the routine genetic diagnostics. An interesting alternative could be RNAseq embracing a qualitative and quantitative analysis of the entire transcriptome in a given tissue. Since all known DOA genes are ubiquitously expressed in accessible tissues such as blood cells or skin biopsies, an RNAseq analysis may well complement the DNA-based genetic diagnostics. Moreover, RNAseq directly assesses the biological effect of those mutations that interfere with transcript processing or secondary adaptive processes. Thus, care should be taken to assess potential down-regulation of mutant transcripts due to nonsense-mediated mRNA decay (Bonifert et al., 2014; Schimpf et al., 2008).

3. The culprit mitochondria

3.1. OPA1 structure and expression

Associating the OPA1 gene with DOA established the first link between a mitochondrial disease and a process not directly related to the respiratory chain. Indeed, OPA1 encodes a protein, which orthologues in the budding (MGM1) (Guan et al., 1993) and fission (Msp1) (Pelloquin et al., 1998) yeasts are involved in mitochondrial membrane fusion and genome maintenance (Alexander et al., 2000; Delettre et al., 2000; Lenaers et al., 2002). This went along with the conserved presence in OPA1, MGM1 and Msp1 protein sequences of a dynamin signature, including a GTPase domain, followed by a middle stalk domain and a coiled-coil GTPase effector domain (GED). The dynamin-type GTPase domain of OPA1 further contains the consensus tripartite GTP-binding motif and the dynamin signature motif. In addition, OPA1 and its orthologues present a mitochondrial targeting sequence (MTS) at the N-terminus required for the import of the protein into mitochondria (Olichon et al., 2002).

As for most other mitochondrial protein, OPA1 is ubiquitously expressed in all nucleated cells. The gene contains 30 coding exons, among which three (4, 4b, and 5b) were initially described as being alternatively spliced, resulting in eight mRNA transcripts and eight protein isoforms (Delettre et al., 2001; Olichon et al., 2007a). The identification of two additional transcript variants (9: NM_001354663, and 10: NM_001354664), containing an extra non-coding exon led to alternative starting codon within exon 3, coding for proteins with a shortened N terminus, which are therefore lacking an MTS and not routed to mitochondria.

OPA1 functions are regulated by posttranslational proteolytic cleavages, expanding the spectrum of isoforms with different molecular sizes and functions (Ishihara et al., 2006). When OPA1 proteins are imported into mitochondria, the mitochondrial processing peptidase cleaves off the first 80 amino-acids corresponding to the MTS, generating the OPA1 long isoform (l-OPA1). This exposes a N-terminal hydrophobic trans-membrane domain (Delettre et al., 2002), and anchors l-OPA1 into the IMM in a type I membrane protein configuration, with the dynamin domains facing the intermembrane space and the cristae lumen (Griparic et al., 2004; Olichon et al., 2002).

Two further mitochondrial proteolytic cleavage sites, S1 and S2, are located downstream of the trans-membrane anchor, in domains encoded by OPA1 exons 5 and 5b, respectively. The constitutive or inducible cleavage at these sites by the mitochondrial peptidase OMA1 (S1) and the i-AAA protease YME1L (S2), respectively, produces shorter soluble forms of OPA1 (s-OPA1), lacking the trans-membrane anchor and are located in the mitochondrial intermembrane space and cristae lumen (Ehres et al., 2009; Griparic et al., 2007; Head et al., 2009; Ishihara et al., 2006; Song et al., 2007). Importantly, the s-OPA1 isoforms might still associate by with the cardiolipin-rich IMM lipid interactions (Ban et al., 2010).

Under physiological conditions, the ratio between the l- and s- OPA1 isoforms is nearly equimolar. Interestingly, l-OPA1 forms that include the domain corresponding to exon 4b are completely processed into s-OPA1 forms (Song et al., 2007). In double knockout YME1L^{-/-}, OMA1^{-/-} cells, l-OPA1 forms are not proteolytically processed (Anand et al., 2014; Wai et al., 2015), suggesting that these two proteases are the major regulators of OPA1 processing. The generation of s-OPA1 forms has also been proposed to be mediated by the presenilin-associated rhomboid-like protease PARL, similar to the situation in yeasts (Cipolat et al., 2006). However, the direct cleavage of OPA1 by PARL remains controversial (Wagener, 2016; Wai et al., 2016). Notably, both SPG7 and AFG3L2 m-AAA proteases regulate YME1L and OMA1 proteases, in a complex networking process that ultimately result in the cleavage of l-OPA1 in s-OPA1 (Consolato et al., 2018; Ehres et al., 2009). This could explain why some dominant SPG7 and AFG3L2 mutations, resulting in fragmented mitochondrial network, can lead to the same clinical phenotype as OPA1 mutations.

3.2. OPA1: a mitochondrial “Swiss army-knife”

OPA1, as a dynamin large-GTPase is forming highly complex homo-polymeric structures, resulting in the formation of cylindrical scaffolds on which is wrapped the IMM, thus establishing a dynamic cristae membrane. In addition, OPA1 is responsible for the IMM fusion in-between two mitochondria engaged in full fusion. These fundamental primary intra- and inter-mitochondrial OPA1 functions are central to the maintenance of many other essential mitochondrial functions, promoting OPA1 as the “swiss army-knife” of mitochondria (Figure 3). Thus, while one of the main functions of OPA1 consists in mediating IMM modelling and fusion, others are responsible for mtDNA maintenance, regulation of respiratory

supercomplex assembly and respiration efficiency, as well as cytochrome c and calcium management. These secondary functions are interconnected as disturbed cristae structure affects respiratory chain which might feedback to mtDNA maintenance; improper IMM fusion might be caused by disturbed cristae structure; lack of fusion affects mtDNA stability, and altered mitochondrial morphology will impact mito-ER contact with downstream consequences on calcium homeostasis. Interestingly, not only all isoforms, but also the processes of generating and balancing those isoforms are integral to this plethora of OPA1 functions. Thus, for optimal mitochondrial function, maintaining a correct complement of OPA1 isoforms is likely important.

3.3. OPA1 and cristae structure and remodelling

OPA1 is the main molecule for maintaining cristae structure and for controlling cristae remodelling (Olichon et al., 2003; Pernas and Scorrano, 2016), independently from its role in IMM fusion (Frezza et al., 2006). Indeed, while knockout or knockdown of OPA1 causes morphologically disorganized cristae (Figure 3) (Cogliati et al., 2013; Frezza et al., 2006; Griparic et al., 2004; Meeusen et al., 2006; Olichon et al., 2003), mild OPA1 overexpression increases cristae stability and tightness (Cogliati et al., 2013; Varanita et al., 2015), whereas strong overexpression is destabilizing the IMM structure, leading to deleterious consequences on the mitochondrial physiology (Olichon et al., 2006). A model has been put forward that l-OPA1 oligomerizes at the cristae luminal membrane and stabilize cristae structure by intercristae membrane tethering (Pernas and Scorrano, 2016). In support of this model, l-OPA1 cleavage or depolymerization by the BCL-2 family member protein BID, results in a widening of the cristae junction, a structure closing the cristae volume in physiological conditions (Frezza et al., 2006; Yamaguchi et al., 2008). Supporting this, the presence of a supplemental coil-coiled domain encoded by exon 5b allows for an additional homo-polymerization step of OPA1, most probably contributing to the cristae junction plug. Consequently, silencing of OPA1 variants including exon 5b leads to the dismantling of cristae junctions, connecting the cristae volume to the inter membrane space, allowing for the release of the cytochrome c in the cytoplasm. This leads to the induction of the irreversible activation of caspases and the engagement in the apoptotic death (Frezza et al., 2006; Olichon et al., 2007b). Thus, by reducing OPA1 amounts or impinging the dynamics of OPA1 homo-polymers, cells become more susceptible to apoptotic stimuli, as observed in DOA cells (Olichon et al., 2007b).

3.4. OPA1 in mitochondrial fusion

The mitochondrial network is in a state of constant rearrangement during the life-cycle of a cell, a process termed mitochondrial dynamics. Depending on the energetic needs, nutrient availability or presence of external stressors, individual mitochondria permanently fuse and divide, leading to homogenization of mitochondrial content across the network. This serves several functions regarding mitochondrial health and capacity: mtDNA distribution, isolation and degradation of defective mitochondria or proteins, adaptation of respiratory capacity and control of apoptosis (Landes et al., 2010). In a healthy state, the balance between mitochondrial fusion and fission controls size, shape and

number of mitochondria to maintain a filamentous network throughout the cytoplasm in most cell types. But slight dysregulation of this delicate balance between fusion and fission can lead to fragmentation of the mitochondrial network, compromised respiratory activity and apoptosis (Figure 3). In addition to OPA1 controlling IMM fusion, two other members of the dynamin family, the mitofusins MFN1 and MFN2 form homo- and heterotypic complexes that catalyse OMM fusion (Ishihara et al., 2004). Notably, l-OPA1 anchored to the IMM interacts with MFN1 to initiate a coordinated fusion of both membranes (Cipolat et al., 2004; Guillery et al., 2008). This occurs through the interaction of OPA1 with a loop of MFN1 located in the intermembrane space (IMS) (Chen and Chan, 2005). OPA1-mediated IMM fusion also depends on IMM potential, and decreased membrane potential will induce the fission of the network due to the cleavage of OPA1 by OMA1 (Kane et al., 2018). IMM fusion of individual mitochondria can be maintained by OPA1 l-forms alone, but overall maintenance of the mitochondrial network dynamics requires the mix of several OPA1 isoforms including the processed s-OPA1 (Anand et al., 2014; Del Dotto et al., 2017; Song et al., 2007). Stoichiometric levels of both l-OPA1 and s-OPA1 are required for efficient and complete fusion of the IMM in vitro, while excess of l-OPA1 or s-OPA1 activates or inhibits fusion, respectively (Ge et al., 2020). Cleavage of l-OPA1 by YME1L is facilitated by high oxidative phosphorylation (OXPHOS) activity and enhances IMM fusion, effectively coupling energy production to mitochondrial fusion. (Mishra et al., 2014). On the other hand, under cellular stress, loss of mitochondrial membrane potential or the accumulation of damaged mitochondrial genomes, l-OPA1 is preferentially cleaved at S1 by OMA1 leading to mitochondrial fission (Baker et al., 2014; Ehses et al., 2009; Head et al., 2009). The OMA1 cleavage of OPA1 blocks fusion, leads to mitochondrial fragmentation and release of cytochrome c from cristae, thus promoting apoptosis. Conversely, depletion of OMA1 changes mitochondrial protein homeostasis and can promote metastatic growth of breast cancer cells (Daverey et al., 2019). Thus, these two mechanisms, initiated by cleavage of l-OPA1 at one site or the other, link the efficiency and health of the individual mitochondrion to the mitochondrial dynamics in general. Damaged or inefficient mitochondria will undergo fission events, promoted by S1 cleavage of l-OPA1 by OMA1, ultimately leading to mitophagic degradation or apoptosis. Healthy mitochondria are maintained by initiating fusion, mediated by S2 cleavage of l-OPA1 by YME1L, preserving and propagating mitochondria that work efficiently. While presence of any OPA1 isoform that can generate l-OPA1 (i.e. isoforms 1, 2, 4 and 7) is sufficient to sustain IMM fusion, cristae structure, mtDNA copy number and super-complex formation in individual mitochondria, regulation of fusion at the level of the entire mitochondrial network seems to require the presence of some s-OPA1 isoforms (3, 5, 6 and 8), since only the presence of both promotes a healthy filamentous mitochondrial network in vitro (Del Dotto et al., 2017). Mitochondrial network fusion is also involved in mitochondrial quality control, a process mandatory to maintain a healthy content of efficient mitochondria. In this process, mitochondrial turnover requires a constant rhythm of OMM fusion and fission, dedicated to the identification of inefficient mitochondria that will be degraded by mitophagy (Shirihai et al., 2015). This further stresses another role of OPA1 related to its involvement

in the fusion process, which is partially disrupted in DOA, with a contributory role in the underlying neurodegenerative process (Kane et al., 2017; Liao et al., 2017; Moulis et al., 2017). The interplay between mitochondrial biogenesis, fusion-fission imbalances and mitochondrial turnover, and how these mechanisms ultimately lead to tissue-specific disease manifestations in the context of neurodegenerative diseases is an area of intense investigations (Fernandez-Vizarra et al., 2011).

3.5. OPA1 and the control of energy metabolism

Cristae structure alterations also directly affect energy production in mitochondria since the components of the respiratory chain and ATP synthase are located here. Within the cristae these individual components can assemble to respiratory chain super-complexes, which are highly efficient in electron transport and ATP production. Knockout of OPA1 has been shown to result in mild defects of oxidative phosphorylation and respiratory chain super-complex assembly (Cogliati et al., 2013; Del Dotto et al., 2017; Jang and Javadov, 2020; Lee et al., 2017; Patten et al., 2014) and in lower enzymatic activity of complex I and IV (Sarzi et al., 2012; Zanna et al., 2008). On the other hand, moderate OPA1 overexpression increases cristae tightness and energetic efficiency by enhancing supercomplex assembly (Cogliati et al., 2013; Varanita et al., 2015). Furthermore, knockdown of OPA1 or MFN1 inhibits oxygen consumption and ATP production, reduces glucose consumption and promotes lactate secretion (Li et al., 2019). Both effects are likely due to the role of OPA1 in maintaining cristae structure and organization, effectively organizing the space where the respiratory chain fulfils its function. Both l-OPA1 or s-OPA1 isoforms alone are able to maintain oxidative phosphorylation function and the mitochondrial respiratory complexes (Del Dotto et al., 2017; Lee et al., 2017), while inter-mitochondrial fusion requires the presence of both l- and s- forms, supporting the notion that OPA1 fulfils separate functions in membrane fusion and in cristae organization (Frezza et al., 2006; Patten et al., 2014).

3.6. OPA1 in the regulation of mitochondrial calcium uptake

Mitochondria are important organelles for Ca^{2+} homeostasis, acting as a major reservoir by taking up large amounts of Ca^{2+} , released from the endoplasmic reticulum (ER). The role of OPA1 in this process is far from trivial as both the structure of the outer and inner membranes are crucial to this process. Indeed, the contact point between the endoplasmic reticulum and the mitochondria, the so-called ER-mitochondrial associated membranes (MAMs), are privileged sites driving a direct loading of the Ca^{2+} released from the ER into mitochondria, while the IMM membrane potential and structure are crucial for the Ca^{2+} uptake and buffering. Nevertheless, experiments performed on RGC and HeLa cells silenced for OPA1 or fibroblasts from DOA patients, showed increased Ca^{2+} uptake into mitochondria, despite loss of the membrane potential (Dayanithi et al., 2010; Fulop et al., 2015; Fulop et al., 2011), while sensitizing cells to excitotoxicity and apoptosis (Dayanithi et al., 2010; Kushnareva et al., 2013).

3.7. OPA1 and the control of apoptosis

The role of OPA1 in cristae structure maintenance impinges not only on bioenergetics but also on apoptosis (Burte et al., 2015). Intrinsic apoptosis coincides with drastic changes in mitochondrial membrane morphologies with fission of the OMM and cristae dismantling, ultimately leading to the release of pro-apoptotic proteins, in particular cytochrome c, from the cristae volume to the inner membrane space, then into the cytosol, leading to the activation of the pro-apoptotic caspases (Pernas and Scorrano, 2016). As soon as an apoptotic process is initiated by high levels of cellular stress, the pro-apoptotic BH3-only proteins BID and BIM are recruited from the cytosol to the OMM. There, they trigger the activation of the pro-apoptotic BCL-2 family members BAX/BAK that form homo-oligomers permeabilizing the mitochondrial outer membrane (Bender and Martinou, 2013). In parallel, BAX/BAK complex activates the metalloprotease OMA1, which in turn cleaves l-OPA1 (Jiang et al., 2014). Hence, disruption of OPA1 complexes results in an opening of cristae junctions toward the intermembrane space, allowing for the complete release of cytochrome c and other proapoptotic factors from the cristae lumen in the intermembrane space, then through the mitochondrial transition pore to the cytoplasm (Cipolat et al., 2006; Cogliati et al., 2013; Frezza et al., 2006; Varanita et al., 2015; Yamaguchi et al., 2008). Cristae junction opening and outer membrane permeabilization are separable events, but both are required for apoptosis (Yamaguchi et al., 2008). Downregulation of OPA1, as observed in DOA increases cellular sensitivity towards apoptotic stimuli and cytochrome c release (Frezza et al., 2006; Griparic et al., 2004; Olichon et al., 2003). OPA1 involvements in apoptosis can therefore be broadly grouped into an inter-mitochondrial role regulating the fusion of multiple mitochondria and an intra-mitochondrial role based on the remodelling of cristae and its consequences on energy production, leading to apoptotic death.

3.8. OPA1 and the maintenance of the mitochondrial genome

Several publications have linked OPA1 mutations responsible for DOAplus cases to disrupted maintenance of the mitochondrial genome and accumulation of mtDNA deletions (Amati-Bonneau et al., 2008; Hudson et al., 2008; Yu-Wai-Man et al., 2010c). Distribution of mtDNA across the mitochondrial network is dependent on mitochondrial fusion, and therefore is proposed to be another task depending on OPA1 (Vidoni et al., 2013). Accumulation of deletions and mutations in mtDNA content is indeed a downstream effect of defective fusion, since mixing of mitochondrial content during fusion helps to dilute defective mtDNA across multiple mitochondria, and favour their elimination by mitophagy, a process requiring mitochondrial fission. Apart from these fusion related effects, there is also a direct route in which OPA1 influences mtDNA maintenance, as this genome is maintained in the matrix of mitochondria in the form of nucleoids anchored to the IMM (Bogenhagen et al., 2008; Holt et al., 2007; Nass, 1969). These nucleoids were found to be anchored to the IMM by a 10kDa peptide resulting from the cleavage of OPA1 isoforms containing exon 4b (Elachouri et al., 2011). This peptide is generated by the cleavage of l-OPA1 isoforms containing exon 4b by Yme1L at the S2 site, resulting in two fragments: the soluble s-OPA1 located into the IMS and a N-terminal part incorporated into the

IMM facing the matrix, strongly anchored into the IMM by two trans-membrane domains, the second one encoded by exon 4b. This fragment, referred to as NT-OPA1-exon4b interacts with the D-loop of the mtDNA, thus attaching the nucleoid to the inner membrane (Yang et al., 2020). Knockdown of OPA1-exon4b isoforms resulted in significantly decreased mtDNA copy number as well as disrupted mtDNA replication and distribution across the mitochondrial network (Elachouri et al., 2011). While this mechanism links NT-OPA1-exon4b to mtDNA nucleoids, the exact mechanism by which anchoring of nucleoids to the IMM facilitates mtDNA replication and maintenance is not yet understood. Thus again, mtDNA maintenance falls into both the intra-mitochondrial role of OPA1, as individual mitochondria maintain and replicate their mtDNA independently, and its inter-mitochondrial role, as the distribution of mtDNA across the mitochondrial network is mediated by inter-mitochondrial fusion, and the elimination of damaged genomes by mitophagy further requires mitochondrial fission.

3.9. Alternative extra-mitochondrial OPA1 functions

As an extra-mitochondrial function of OPA1, the proposed localization of OPA1 on lipid droplets, and its function as an A-kinase anchoring protein controlling lipolysis (Pidoux et al., 2011; Rogne et al., 2018), remains controversial (Belenguer and Pellegrini, 2013). Interestingly, two recently identified OPA1 transcripts encoding isoforms 9 and 10 of OPA1 are lacking the mitochondrial targeting sequence and therefore could play a role in this proposed mechanism.

3.10. Functions of the other genes involved in DOA

Since the initial discovery of OPA1 in 2000, all genes that were identified as causing DOA are related to effective mitochondrial membrane dynamics, with the exception of SSBP1 and NR2F1. Indeed, MFN2, AFG3L2 and SPG7 are encoding proteins regulating the mitochondrial fusion, either by acting on the outer membrane, as MFN2 in concert with OPA1, or by regulating the cleavage process of OPA1, as AFG2L2 and SPG7 (Caporali et al., 2020; Charif et al., 2020), through the processing of OMA1 and YME1L. In the same line of evidence, but with an opposite effect, OPA3 and DNM1L are encoding actors of mitochondrial fission, OPA3 most probably acting on the inner mitochondrial membrane (Ryu et al., 2010), whereas DRP1 pinches both membranes simultaneously by tightening the OMM (Smirnova et al., 1998). These observations emphasize that mitochondrial network dynamics and the equilibrium between fusion and fission events are of utmost importance to maintain RGCs physiology. This concept can further be extrapolated to WFS1 pathophysiology (Angebault et al., 2018; Cagalinec et al., 2016; La Morgia et al., 2020), which is related to the transfer of calcium from the endoplasmic reticulum to the mitochondria, a process which is intimately related to the MAM contact points between these two organelles and the role of mitochondrial dynamics, both being controlled in particular by MFN2 protein (de Brito and Scorrano, 2008).

Concerning SSBP1, it was recently demonstrated that the pathological mechanism associated with the identified missense variants is related to a loss of integrity of the mitochondrial genome, leading to a

significant decrease of mtDNA copy number (Del Dotto et al., 2020; Jurkute et al., 2019a; Piro-Megy et al., 2020). This could be related to the involvement of OPA1 in the maintenance of the mtDNA integrity, although no mtDNA depletion has been reported in OPA1 patients; on the contrary a mild increase of mtDNA copy number was found in lymphocytes from DOA patients (Sitarz et al., 2012).

Concerning NR2F1, its involvement as a transcription regulator of the brain development, eventually activated by steroid and thyroid hormones, suggests that the pathophysiological mechanism causing DOA is most probably occurring early during embryogenesis (Bertacchi et al., 2019), while involving potential mitochondrial dysfunctions, as both steroids and thyroids can regulate mitochondrial transcription (Psarra and Sekeris, 2008), a process that has never been associated yet to OPA1 or any other gene involved in DOA.

Thus altogether, the main pathophysiological processes involved in DOA are related to the IMM and OMM shaping dynamics, while few additional processes are emerging with the recent discovery of new causative genes.

4. The pathophysiology of DOA

4.1. Why RGCs and the optic nerve degenerate in DOA?

When considering an inherited mitochondrial disease, one crucial point is its tissue and cell-specificity. While the mitochondrial defects are found in all nucleated cells of the body, the consequences of this mitochondrial damage are only visible in a certain tissue or cell type. This observation is also the main conundrum for DOA, with respect to mitochondria in RGCs and the optic nerves.

RGCs are neurons, with their soma located in the most inner layer of the retina, and their dendrites establishing a network of complex connections in the inner plexiform layer to contact the bipolar and amacrine cells. RGC axons are running all along the inner retinal surface to converge to the optic disk, pass through the lamina cribrosa (LC), and enter the optic nerve (ON). Then, they pass through the optic chiasma, run along the optic tracts, to ultimately establish their synapses in the lateral geniculate nucleus. Physiologically, to prevent light scattering and allow highly visual performances, RGC axons are not myelinated in the eye, thus requiring tremendous amounts of energy to transduce the action potentials. Once they pass the LC, RGC axons become myelinated and transduce the action potentials in an energy sparing mechanism based on a saltatory propagation. Additionally, RGC axons are quite long, measuring 5 to 8 cm in humans, and therefore represent more than 99 % of the RGC total volume. Independent of the visual stimulus, they discharge approximately 15 action potentials per second, thus imposing extremely high energy requirements that must be fulfilled by mitochondria all along the axon, in two strongly contrasting environments: in the eye and in the optic nerve (Yu et al., 2013).

Analyses of mitochondrial distribution in RGCs, have revealed an uneven distribution of mitochondria in mammals, with a dense concentration in the unmyelinated part of the axon, eventually forming varicosities aggregating mitochondria, while mitochondria are getting more diffused in the myelinated

part of the axon (Figure 4) (Bristow et al., 2002; Yu Wai Man et al., 2005). Notably, the shift between plentiful and scarce mitochondrial abundance is occurring precisely at the LC, with a tight correlation to the myelination status of the axon (Barron et al., 2004). Electron microscopy images of the mouse optic nerve confirmed these observations. Indeed, they revealed that mitochondria were round with a clear matricial content in the unmyelinated part of the axon, while they are elongated with a dark matricial content in the myelinated part of the axon (Figure 5). Notably, images focusing on the LC, where both unmyelinated and myelinated axons co-exist, revealed that the round clear mitochondria are getting associated towards fusion, or *vice versa*, that long dark mitochondria are getting prone to divide, according to the status of myelination of the axon in which they are located. These very precise observations at the LC emphasize the major membrane dynamics modifications occurring during the mitochondrial axonal transport at the LC, involving both intra and inter mitochondrial membrane modifications, mirroring the basic functions of OPA1.

They further highlight a crucial correlation between mitochondrial dynamics and myelination, a process for which the molecular mechanism remains unknown, but highly relevant to neurological diseases related to demyelination. In this respect, disturbance in mitochondrial dynamics has been linked to the development of neurodegenerative disorders such as Parkinson and Alzheimer diseases (Burte et al., 2015; Corti et al., 2011; Enciu and Popescu, 2013), while LHON disease is often associated with demyelination and clinical, neuroradiological and serological changes indistinguishable from multiple sclerosis (MS) (Harding et al., 1992; Pfeffer et al., 2013). In addition, an MS-like illness has also been described in DOA patients with confirmed pathogenic OPA1 mutations (Verny et al., 2008). Thus, in view of the overlapping clinical and pathophysiological features seen in DOA, LHON and other central nervous system diseases, the link between mitochondrial dynamics, demyelination and the development of MS highlights a pathological pathway that is therapeutically relevant.

4.2. Why specifically the RGCs from the papillo-macular bundle degenerate in DOA?

As mentioned in the description of the visual field alterations found in clinical examinations of DOA individuals, disclosing systematically a central scotoma, a further concern consists in explaining why the small parvo RGCs located at the centre of the retina preferentially degenerate in the course of the disease.

From the former observations the question is emerging of how mitochondria are fuelled in the two different regions of the RGC axon. If the optic nerve glia provides lactate to the axons in the optic nerve (Funfschilling et al., 2012; Magistretti and Allaman, 2015), which is then dehydrogenated into pyruvate to feed the Krebs cycle and the mitochondrial respiration, the situation is less evident in the intra-ocular unmyelinated axonal region. Indeed, inside the eye, it remains unclear which carbohydrate is available and which cells contribute to the fuelling of the unmyelinated axon to produce efficiently the amounts of ATP required to sustain the high frequency of action potentials (Winkler et al., 2004). This fuelling

aspect is of further concern in the macular region, where the inner retinal vascular network becomes progressively restricted while converging to the fovea. Notably, angiography OCT examinations of DOA patients revealed an altered vascularization network in the papillo-macular bundle of the retina (Balducci et al., 2017), which might interfere with the availability of carbohydrates and oxygen to fuel RGCs mitochondria that are already compromised by OPA1 mutations in their efficiency to produce ATP.

A further concern is the permanent exposure of the central retina to the oxidative stress generated by the light propagating through the eye. It is indeed well known that low frequency wave-lengths in the range of 400 to 480 nm, are of peculiar stress for RGCs survival and mitochondrial efficiency (Osborne et al., 2017). Thus combined with the mitochondrial defects related to OPA1 mutations, associating lower energy efficiency to a higher ROS production, damages induced by light exposure might contribute to the increased degeneration of RGCs located in the central retina.

Finally, these very same RGCs located in the central retina that preferentially degenerate in DOA and LHON, are small parvo - or midget - RGCs with an axon diameter less than one micron. It is known that these parvo RGCs transduce the visual information at a lower rate, than the magno - or parasol - RGC located more in the periphery, which have wider axons (Perge et al., 2009). Although this correlates to lower energy requirements for transducing the central visual information, the small diameter of parvo RGCs might impair the mitochondrial transport, as they cannot accommodate more than one mitochondrion per cross-section, thus imposing specific constraints to the mitochondrial network shape during the axonal transport in parvo RGCs (Pan et al., 2012).

Although, none of these hypotheses have been challenged experimentally today, due to the lack of relevant animal models reproducing a central scotoma, each one alone, or their combinations could contribute to the highly specific pattern of RGC degeneration in the centre of the retina, as observed in DOA patients (Figure 6).

5. Animal models of DOA

Since the discovery of OPA1 and its connection to DOA, considerable efforts went into the generation and validation of animal models of this disease, to address the pathophysiological mechanism responsible for RGC degeneration, and open opportunities to evaluate treatments. To date, mouse, zebra fish, fly and worm models of DOA are available and are being actively studied.

5.1. Mouse models of DOA

Mouse and human OPA1 are highly conserved with an overall 96 % identical amino acid sequence suggesting mouse models of DOA might faithfully replicate human disease. In this regard, one relevant difference between mouse and human OPA1, is the number of different OPA1 splice variants. While alternative splicing of exon 4, 4b and 5b in humans gives rise to eight different OPA1 isoforms located

into mitochondria, in mouse only four different Opa1 isoforms are produced, resulting from the alternate splicing of exon 4b and 5b only (Akepati et al., 2008). As balanced production of different OPA1 isoforms is important for its proper function (Del Dotto et al., 2018a), the difference of OPA1 patterns between mouse and human might impact the performance of DOA mouse models in replicating human disease, and should be considered when using these models for testing potential treatments, in particular gene therapy (Figure 7).

So far, four mouse models impairing Opa1 function have been generated, with three used in retinal research and one applied mainly for metabolic research (Rodriguez-Nuevo et al., 2018; Zhang et al., 2011). Two of the models, Opa1^{+/-del} (Alavi et al., 2007) and Opa1^{+/-STOP} (Davies et al., 2007) originate from an ENU mutagenesis screen, while the third one, Opa1^{+/-delTTAG} (Sarzi et al., 2012), was generated by knocking-in a mutated version of exon 29, reproducing the most frequently encountered mutation in human. The fourth model (Zhang et al., 2011) contains two lox P sites flanking Opa1, allowing for the complete deletion of Opa1 in any cells or organs expressing the cre-lox nuclease to invalidate both Opa1 alleles. This latter model is useful for studying the consequences of cell-restricted complete loss of Opa1, since homozygous deletion of Opa1 is embryonic lethal (Alavi et al., 2007; Davies et al., 2007; Sarzi et al., 2012). DOA being caused by mutations either leading to a OPA1 gain-of-function or haplo-insufficiency, Opa1^{+/-del}, Opa1^{+/-STOP} and Opa1^{+/-delTTAG} mice can be considered *bona fide* models for DOA related to haplo-insufficiency, and will be described here.

Opa1^{+/-del} mice carry the intronic point mutation c.1065+5G >A, causing an alteration of a splice donor sequence of exon 10. This leads to the skipping of exon 10 during splicing, and results in an in-frame deletion of 27 amino acids in the GTPase domain of Opa1. Although the mutant Opa1 mRNA is intact and detectable by RT-PCR at wildtype levels, the resultant protein is most probably unstable and immediately degraded, reducing Opa1 protein levels by approximately 50 % (Alavi et al., 2007).

A non-sense C to T transition at the beginning of Opa1 exon 8, leading to a premature stop codon (p.Q285X) is the basis for the Opa1^{+/-STOP} model of DOA (Davies et al., 2007). In contrast to Opa1^{+/-del}, Opa1^{+/-STOP} animals have lower levels of total Opa1 mRNA, about 50 % of littermate controls (our unpublished results). The presence of a newly introduced premature termination codon strongly suggests degradation of mutant Opa1 mRNA by nonsense-mediated mRNA decay as the basis for the observed Opa1 haploinsufficiency.

Finally, the Opa1^{+/-delTTAG} model was generated by targeted deletion of the TTAG sequence in Opa1 exon 29, generating a frameshift causing the loss of the last 58 amino acids in all OPA1 isoforms and variable tissue-specific reduction of overall Opa1 protein levels. Interestingly, truncated Opa1 isoforms were not readily detectable by Western blot analysis suggesting either mRNA decay or rapid degradation of the truncated Opa1 protein (Sarzi et al., 2012).

Thus, three DOA mouse models, $Opa1^{+/del}$, $Opa1^{+/STOP}$ and $Opa1^{+/delTTAG}$, are driven by $Opa1$ haploinsufficiency rather than a dominant-negative effect of mutated $Opa1$ protein. While non-sense mediated mRNA decay likely precludes the production of any $Opa1$ protein, in the case of in-frame deletion as in $Opa1^{+/del}$ or short C-terminal truncation as in $Opa1^{+/delTTAG}$, it is conceivable that mutated $Opa1$ protein might exert a dominant-negative effect in the time window between translation and protein degradation. These differences in how haploinsufficiency is caused - accelerated protein degradation in $Opa1^{+/del}$, non-sense-mediated mRNA decay in $Opa1^{+/STOP}$ and either one of these pathways in $Opa1^{+/delTTAG}$ – might explain the differences in the phenotypic expression of DOA as detailed below.

5.2. Characterization of the $Opa1^{+/del}$ mouse model of DOA

Initial characterization of the $Opa1^{+/del}$ mouse revealed the importance of $Opa1$ for embryonic development, as homozygous mutant mice died in utero around E8.5, while heterozygous mutant mice had normal outward appearance (Alavi et al., 2007).

Comparing $Opa1^{+/del}$ with wild-type animal at 2 and 9 month, neither gross changes in the optic nerve fibre layer or retinal degeneration as assessed by scanning laser ophthalmoscopy, nor abnormal ERG responses were found. Similarly, histological analysis of 2 and 4-month-old $Opa1^{+/del}$ animals did not reveal any difference in the retinal architectures. In 13-month-old $Opa1^{+/del}$ animals compared to wild-type littermates, however, a slight reduction of RGCs became evident upon histological analysis. Retrograde labelling of RGCs in $Opa1^{+/del}$ animals revealed a 50 % reduction of labelled RGCs at 13 months of age compared to controls. Overall, degeneration of RGCs was variable in these $Opa1^{+/del}$ animals and occurred rather late. Phenotypic variability is also a hallmark of DOA in humans and can be attributed to modifier effects. This may hold also true in $Opa1^{+/del}$ animals, as this line is kept and cross-bred on a mixed C3H-C57/Bl6 background, with the potential for differences in $Opa1$ expression levels or heterogeneous genetic backgrounds.

An in-depth analysis of RGC subgroups and their loss in aged $Opa1^{+/del}$ animals (Gonzalez-Menendez et al., 2015) revealed the preferred loss of small calibre RGC axons. About 95 % of lost axons were smaller than 0.4 μm^2 at 18 months of age. Interestingly, intrinsically photosensitive RGCs (ipRGCs) were spared in $Opa1^{+/del}$ animals, even in the absence of melanopsin expression. Why ipRGCs are significantly less sensitive to $Opa1$ haploinsufficiency and many other stimuli (Fogo et al., 2019; Perez de Sevilla Muller et al., 2014) compared to non-melanopsin expressing RGCs remains unclear, but mirror the absence of circadian alteration in DOA patients. The large calibre of 0.632 μm (lower limit: 0.113 μm , upper limit: 1.455 μm) of the ipRGCs axons, might however answer this question, at least for the $Opa1^{+/del}$ animals (Kim et al., 2019). Why large calibre axons might be beneficial for $Opa1^{+/del}$ RGCs is unclear, but one might speculate that impairment of axonal transport might be less pronounced, or that impaired mitochondrial energy production might be better compensated in larger calibre axons, due to their larger volume to surface ratio. In addition to outright axonal and cell loss, RGCs from

Opa1^{+/-del} animals also displayed electrophysiological dysfunctions (Gonzalez-Menendez et al., 2015). Indeed, multi-electrode array recording of whole mount retinas revealed differences in spatial and temporal tuning of RGCs from Opa1^{+/-del} animals, compared to wildtype controls. Using visually evoked potential (VEP) measurements, a visual function deficit in Opa1^{+/-del} animals was ascertained (Heiduschka et al., 2010). While ERG recordings were not altered in these animals compared to controls, VEP displayed significantly reduced amplitudes with unchanged latency. As electroretinogram (ERG) measures overwhelmingly the electrical activity of photoreceptors as well as bipolar and amacrine cells, the unchanged ERG in Opa1^{+/-del} animals compared to controls highlights the highly selective effect of Opa1 mutation in the retina on RGC function and survival, but not on any other retinal cell types.

Consistent with the loss of retrograde labelling, optic nerve ultrastructural analysis revealed major loss of axons, membrane whorls and diminished myelination in Opa1^{+/-del} animals. An in-depth analysis of extra-ocular phenotypes of aged Opa1^{+/-del} animals revealed an increased tendency for upper limb tremor and an abnormal clutching reflex indicative for neuromuscular deficits in mutant animals (Alavi et al., 2009). Interestingly, upper limb tremor developed preferentially in male compared to female animals. When studying the running and training capacity in Opa1^{+/-del} mice, greatly increased endurance capacity was observed in mutant mice compared to controls (Caffin et al., 2013). This increased endurance was observed despite their inability to increase mitochondrial biogenesis, usually accompanying endurance training. Finally, in a unique adaptive strategy, Opa1^{+/-del} mice shifted their metabolism towards higher utilization of fatty acids compared to control animals, witnessing a metabolic shift towards oxidative phosphorylation (OXPHOS) mitochondrial respiration.

5.3. Expression of DOA phenotypes in the Opa1^{+/-STOP} mouse model

Initial characterization of 6-month-old Opa1^{+/-STOP} animals revealed no significant retinal pathology upon funduscopy comparable with the human situation (Davies et al., 2007), while examination of the optic nerve by electron microscopy revealed significant abnormalities starting at 9 months of age, presenting gross whorls of myelin together with an overall loss of myelination of optic nerve fibre bundles (Davies et al., 2007). Consistent with this observed optic nerve damage, Opa1^{+/-STOP} animals display reduced visual acuity in optokinetic drum testing and altered sensitivity to light onset in activity measurements on the running wheel (Davies et al., 2007). While the number of retinal ganglion cells in Opa1^{+/-STOP} mice was not reduced with age compared to controls, increased dendritic pruning of ON-centre retinal ganglion cells was reported, and likely explains the observed visual impairment in these animals in the absence of outright RGC loss (Williams et al., 2010). A study of Opa1^{+/-STOP} animals over 24 months revealed disorganization of RGC myelination in the absence of decreased axonal density (White et al., 2009). This optic nerve damage was accompanied by the increased presence of mitochondria with opaque matrix, as detected by electron microscopy, and significantly elevated levels of autophagy in mutated RGCs, compared to controls. Further electrophysiological analysis of Opa1^{+/-STOP} animals confirmed impaired visual function. Under scotopic conditions, single flash stimuli across the full range

of intensity did not reveal any significant difference in a- and b-wave amplitude nor in VEP, between mutant and control animals. However, under photopic conditions, ERG revealed a significant depression of the photopic negative response amplitude in $Opa1^{+/STOP}$, compared to control animals. Similarly, alteration in the VEP in $Opa1^{+/STOP}$ animals with a reduction in the positive deflection (P2 wave) compared to controls were recorded (Barnard et al., 2011).

Beyond visual function, primary SHIRPA screening of 37 different health and neurological parameters in 6-month-old animals pointed to neurological abnormalities of $Opa1^{+/STOP}$ animals. Mutant animals showed reduced activity in the viewing jar test, lower locomotor activity and increased biting upon provocation. Of note, hearing was not impaired in $Opa1^{+/STOP}$ animals compared to controls. Initially at 6 months of age, no gross impact of the $Opa1^{Q285X}$ mutation on heart and skeletal muscle histology was observed (Davies et al., 2007). However, a more detailed analysis of cardiac function revealed late-onset cardiomyopathy at 12 months of age, with impaired function of cardiac mitochondria, coinciding with decreased fractional shortening, cardiac output, and myocyte contraction (Chen et al., 2012). On the cellular level, mitochondria in muscle explants from $Opa1^{+/STOP}$ mice were predominantly fragmented compared to wild-type littermate controls, displaying a normal, interconnected network in muscle explants. Consistent with this observation, mitochondrial respiration and specifically levels of complex IV subunits were diminished in fibroblasts from $Opa1^{+/STOP}$ animals (Kushnareva et al., 2016).

5.4. The $Opa1^{+/delTTAG}$ model of DOA

While above mentioned $Opa1^{+/del}$ and $Opa1^{+/STOP}$ animals are more reminiscent of non-syndromic DOA with a mainly visual phenotype, $Opa1^{+/delTTAG}$ mice resemble syndromic DOA, characterized by additional extra-ocular disease, such as hearing loss and other neuromuscular dysfunctions (Sarzi et al., 2012). Age-dependent analysis of RGC number revealed a 40 and 50 % loss of these cells in $Opa1^{+/delTTAG}$ at 9 and 16 months, respectively. While ERG responses were not affected by the $Opa1^{delTTAG}$ mutation, VEP measurement showed a progressive increase in latency reaching significance at 13 months of age. These observations are consistent with RGC damage due to impaired $Opa1$ function. In line with this finding, demyelination of optic nerve fibers and axonal degeneration were already evident at 5 months of age in these mice, as was the presence of extra-ocular phenotypes like progressive hearing loss. While mutant animals had normal hearing at 3 months of age, perception of high frequencies was impaired at 5 months of age, with severe deafness at 11 months of age. Interestingly, the sciatic nerve also suffered in these animals and showed signs of demyelination and increased autophagy as early as 5 months of age, indicative of peripheral neuropathy. Furthermore, locomotive abilities were reduced, starting at 5 months of age, with impaired hind paws strength affecting male and female differently. On the cellular level, already at 5 months of age a decrease in mitochondrial electron transport chain complex IV activity was reported in the retina, which at 11 months of age was significant not only in the retina but also in the optic nerve and glycolytic muscle. Interestingly, oxidative muscles such as heart or soleus did not exhibit this diminished drop in complex

IV activity. Nonetheless, electron microscopic analysis of heart muscle from 5-month-old Opa1^{+/-delTTAG} animals revealed ultrastructural abnormalities in mitochondrial organization. This coincided with a significant 50 % loss of mitochondrial DNA content in heart muscle tissue. Detailed studies revealed a sex-dependent impact of the Opa1^{+/-delTTAG} mutation, with female animals affected earlier and with higher penetrance. Impairment of visual function in female Opa1^{+/-delTTAG}, as measured by VEP latency, preceded by four to seven months that in male animals. Similarly, optomotor response was affected significantly in female Opa1^{+/-delTTAG} animals, already at 5 months of age, while their male counterparts were not significantly affected at this time point. Pregnenolone, a steroid precursor, was reportedly increased in female Opa1^{+/-delTTAG} animals. Interestingly, lowering sex hormones through ovariectomy did blunt this effect suggesting a role for steroid dysregulation in the development of DOA (Sarzi et al., 2016). In line with these observations, metabolomics analysis revealed a specific metabolic signature for optic nerves from symptomatic female Opa1^{+/-delTTAG} animals, at 3 months of age, well before the first ocular manifestations.

Altogether, the three Opa1 mouse models present with varying severity, penetrance as well as time of onset, like in DOA human situations, characterized by variable age of onset of the disease, incomplete penetrance and differential phenotypic expression. Keeping this in mind, the three Opa1-DOA mouse models replicate most aspects of the human disease and are thus very useful to understand the disease itself and most importantly, are well suited for developing and testing novel therapeutic approaches to tackle the currently unmet medical needs of DOA.

5.5. Modelling DOA in other organisms

OPA1 is highly conserved across the animal kingdom and fungi with orthologues found in fish, fly, worm, and also yeast. While not explicitly used to model DOA, Opa1 models are very useful for understanding the functions of this gene and the associated dysfunctions related to Opa1 modifications. Using morpholino-mediated knockdown of zebra-fish opa1 (Rahn et al., 2013), a critical role for opa1 in development of heart, and also the eye and the nervous system was confirmed, as downregulation of opa1 during development caused heart abnormalities and retarded eye growth, and upon behavioural testing, startle response, locomotion and swim duration were further negatively impacted. These observations are in line with the above findings in mouse models, also displaying deficits in locomotion, hearing and neurological development.

In the fly *Drosophila melanogaster*, insertion into the 2nd exon and the 3rd intron of dOPA1 resulted in decreased dOpa1 protein levels consistent with haploinsufficiency. Heterozygous flies did not display any observable gross eye phenotype. But further dOpa1 downregulation modified the spatial density of mitochondria, changed their spatial distributions and morphology, while impairing their axonal transport (Yu et al., 2016). This resulted in an ocular phenotype in form of rough/glossy eyes, due to increased apoptosis of lattice cells, impacting ommatidia organization and diminished deposit of lens and pigment

material (Yarosh et al., 2008). Thus, results from studying OPA1 in non-mammalian animal models supports and further highlights the importance of OPA1 for proper mitochondrial functions and distribution across species, and emphasizes OPA1 involvement in eye development and function.

6. Metabolomics considerations in DOA

Besides the fundamental and crucial involvement of mitochondria in producing ATP, mitochondria have retained many original metabolic pathways from their bacterial ancestors that are interconnected with the whole cell metabolism. This concerns in particular the synthesis of lipids, amino-acids, nucleotides and steroids, and can be technically investigated by mass spectrometry, using targeted and untargeted metabolomics approaches. How these pathways are dysregulated in mitochondrial diseases is a recent emerging topic of investigations, which has been extensively addressed for OPA1-related DOA, using cell models, animal organs and human plasma.

6.1. Qualitative aspects

Cellular analyses were performed on *Opa1*^{-/-} mouse embryonic fibroblasts (MEF), which were further manipulated to re-express different OPA1 pathogenic alleles responsible for clinical presentations with different severity, or on rat cortical neurons silenced for *Opa1*. Results disclosed signatures essentially involving amino acids and nucleotide variations, as well as phospholipid remodelling (Figure 8). In particular, aspartate and glutamate concentrations were significantly decreased, with potential deleterious consequences on the nucleotide metabolism, while other amino-acids and secondary amines, like spermine, spermidine and taurine, had variable evolution of concentrations according to the cellular model, reflecting a potential ER stress condition and the activation of a proteostasis mechanism (Bocca et al., 2018a; Chao de la Barca et al., 2019; Chao de la Barca et al., 2020). Of note, modifications of the nucleotides and NAD synthesis, and of creatine and phospho-creatine were also found, which might result from the deficiency of the respiratory chain and energy storage (Bocca et al., 2018b). Phospholipids were also significantly affected, in particular sphingomyelin, phosphatidylcholines and lysophosphatidylcholines, which were increased in *Opa1*^{-/-} cells, witnessing a major adaptation of membrane composition that could parallel the reduced mitochondrial dynamics, and also alterations in the bio-synthesis of myelin. Further changes in ceramides, gangliosides and phosphatidylinositols were evidenced in the *Opa1*^{-/-} MEF signature, reflecting their susceptibility to apoptotic signalling (Bocca et al., 2019; Chao de la Barca et al., 2019; Chao de la Barca et al., 2020). Numerous triglycerides were also consistently increased in the *Opa1*^{-/-} cells (Bocca et al., 2019) with fold changes up to 18, although abnormal triglycerides concentrations have never been reported in DOA patients, and were not found in the plasma of the *Opa1*^{+delTTAG} mouse model (Chao de la Barca et al., 2017). This triglycerides accumulation in *Opa1*^{-/-} MEFs was shown to result from an impaired fatty acids flux due to reduced mitochondrial uptake and oxidative metabolism. Analysis of the plasma from 11 months aged *Opa1*^{+delTTAG} mice revealed an increase in several phospholipids while decreased concentrations of amino acids and sarcosine. This paralleled a specific signature of the optic nerve, involving altered

concentrations of phospholipids, amino acids, acylcarnitines and carnosine, reflecting the degenerative process and the glial scars occurring at that time in the optic nerve. A highly different optic nerve signature was found at 3 months of age, including decreased sphingomyelins and lysophosphatidylcholines, reflecting the myelin sheath progressive disorganisation, and changes of neuroprotective metabolites such as dimethylarginine, carnitine, spermine, spermidine and carnosine, witnessing concomitant metabolic dysfunctions (Chao de la Barca et al., 2017). Ultimately, plasma from 25 OPA1 patients were compared to age and sex matched controls (Bocca et al., 2018b). Results indicated major changes of the purine metabolism, including xanthine, hypoxanthine and inosine, and again decreased concentrations of aspartate and glutamate. Furthermore, a significant reduction in nicotinamide concentration, more pronounced in DOA+ than in isolated DOA, was evidenced, converging with the change in NAD concentration found in Opa1^{-/-} MEFs (Bocca et al., 2018a).

6.2. Quantitative aspects

Beyond the original metabolomics signatures that disclosed focuses on DOA pathophysiology, some fascinating quantitative and chronological aspects have emerged from these analyses. First, the metabolomics study of the Opa1^{+/-delTTAG} mouse model (Chao de la Barca et al., 2017), revealed that among the 10 organs analysed at 11 months of age, only the optic nerve and the plasma showed a significant signature compared to wild-type organs, thus highlighting the specificity of the disease and the role of the metabolism in its pathophysiology. Second, the metabolomics signatures at 11 months were only significantly different between female cohorts, but not between males, thus pointing to a gender effect that was further confirmed by studying steroid biosynthesis in the same mouse model (Sarzi et al., 2016). Third, at three months of age, only the optic nerve showed a significant metabolomics signature, compared to wild type animals, suggesting that important metabolic dysfunctions occur well before the first ophthalmologic dysfunctions that were evidenced at 5 months in this DOA model (Chao de la Barca et al., 2017). Lastly, the analysis of the metabolomics signatures of human OPA1 missense pathogenic variants expressed in Opa1^{-/-} MEFs, identified quantitative changes proportional to the severity of the clinical presentation associated to each mutation (Chao de la Barca et al., 2020), thus identifying predictive biomarkers of the disease, and emphasizing the high resolving power of metabolomics in hierarchizing OPA1 missense mutations, according to their clinical expressivity. The most discriminant metabolic features associated with clinical severity were hydroxyproline, the spermine/spermidine ratio, amino acid pool and several phospholipids, emphasizing proteostasis, endoplasmic reticulum (ER) stress and phospholipid remodelling as the main mechanisms ranking OPA1 allele impacts on metabolism.

It further suggests that by restoring physiological concentrations of some of the decreased compounds by supplementation treatment, such as with nicotinamide, one might expect to improve the condition of OPA1 patients.

7. Conclusions and future directions towards therapies

While we came a long way since the initial description of *OPA1* mutations and their link to DOA, DOA pathophysiology still remains an active field, requiring further understanding of the pathomechanisms that affect the RGCs of DOA patients early in life. Nevertheless, we have reached a point from which pre-clinical and clinical therapeutic trials can be designed and assessed. The biological models ranging from cell lines and mouse models, to well-characterized DOA patient cohorts, are now available for efficacy assessment of potential therapies. Also, we have amassed discriminant molecular, cellular, and histological criteria as well as ophthalmological parameters characterizing RGCs integrity along the disease evolution, to assess the patient benefits of a therapeutic strategy. In addition, the original situation of DOA at the intersection between ophthalmology, neurology and mitochondrial diseases, allows to consider treatment strategies either based on gene therapy, now tested in humans for many ophthalmological genetic diseases, or pharmacological therapies that are the best ways to address non-retinal mitochondrial and neurological diseases, and on nutritional therapies that can be inferred from the recent metabolomics data. With this armada of opportunities, we can either aim at restoring a physiological level of wild-type *OPA1* mRNA and protein expressions, or target the molecular and cellular consequences induced by *OPA1* mutations.

7.1. Gene therapy for DOA

The first objective involves the concept of gene therapy. Intuitively, the best approach would be to restore a wild-type gene by correcting the morbid mutation. Much hope is now focused on the CRISPR-Cas9 tool that can be targeted into RGCs with a viral vector, as the AAV2, or transfected as an mRNA, together with the guide RNA to trigger recombination at the *OPA1* locus. Although this is conceptually accessible with the molecular tools available nowadays, special attention should be paid on the possible off-target effects that would randomly jeopardize the efficiency of the initial objective. Another solution consists in expressing *OPA1* from a transgene, after delivering a therapeutic plasmid into RGCs. This approach has been tried in the *Opa1*^{+/~~delTTAG~~} mouse model, using the human *OPA1* isoform 1 cDNA, which includes exon 4 but not exons 4b and 5b. Although encouraging, results did not amount to a proof of concept required for a transfer in humans (Sarzi et al., 2018). Indeed, many concerns came out from these experiments, because this approach does not allow controlling *OPA1* expression levels in transfected RGCs. As the increase from 50 % to 100 % of *OPA1* protein level is crucial to restore the mitochondrial physiology, approaches involving uncontrolled *OPA1* expression could be deleterious. Indeed, *OPA1* over-expression leads to unbalanced hyper-fusion of the mitochondrial network, thus reproducing the cellular phenotype associated to *DNM1L* and *OPA3* mutations. In addition, the restriction associated to the expression of a single therapeutic *OPA1* isoform, limits the process, as the pattern of expression of all *OPA1* isoforms would be out of balance, with deleterious consequences on the ratio between l- and s-*OPA1* isoforms. Thus alternative strategies are required first to by-pass these limitations and second to address both haploinsufficient and dominant negative mutations.

An alternative solution to address DOA gene therapy is taking advantage of the remaining, still functional copy of the *OPA1* gene in the haploinsufficient condition encountered in the majority of patients. While it is unclear why the functional allele is unable to compensate for the diseased allele under physiological conditions, it might still be possible to boost expression from this healthy allele. Increasing expression from the healthy allele by a factor of two would negate the loss of the diseased *OPA1* allele, thus offering a functional cure. Here, artificial transcription factors (ATFs) come into play. ATFs can be constructed from a DNA-binding domain capable of recognizing a suitable DNA sequence inside the promoter of a gene of interest and a transcriptionally active domain capable of suppressing or activating gene expression such as KRAB and SID or VP64 (Segal et al., 1999). For the DNA-binding domain, programmable or otherwise customizable options in the form of nuclease-dead Cas9 (dCas9) (Perez-Pinera et al., 2013), transcription activation-like effectors (TALE) (Sanjana et al., 2012) or modular zinc finger proteins are available. There is an inverse correlation between the ease of generating an ATF using these customizable DNA-binding domains and their size. While dCas9 are targeted simply by providing a suitable guide RNA, TALE-based activators or zinc finger proteins (ZFPs) necessitate an involved cloning or lengthy screening procedure, respectively. However, to address a 20 base pair (bp) DNA sequence, dCas9 uses about 1350 and TALE-based activators about 1000 amino acids, while zinc finger proteins are capable of engaging an 18 bp DNA target with around 180 bp. Thus, for use in a gene therapeutic setting and with delivery of a therapeutic ATF expression construct via AAV in mind, TALE and ZFP based transcriptional activators for boosting expression of *OPA1* in DOA patients might be useful. Expression cassettes for TALE and especially ZFP-based ATFs are small enough to fit into the size budget afforded by the limited packaging capacity of current AAV-derived therapeutic vectors without severely hampering the choice of promoter driving expression in the target cell.

Delivery of activating ATFs via AAVs might be seen simply as an alternative to gene replacement therapy. In contrast to gene replacement, however, where not a full gene but a cDNA is delivered, ATFs allow for the restoration of full-level gene expression from a genomic locus. Thus, for a gene where alternative splicing leads to the production of several mRNAs variants and multiple protein isoforms, application of ATFs would increase gene output, while maintaining protein expression pattern. In the case of DOA, ATFs would be capable of maintaining the important balance between l- and s-*OPA1* isoforms.

While ATFs are promising candidates for the treatment of DOA, some caveats remain. First and foremost, treatment is limited to patients suffering from *OPA1* haploinsufficiency. Increasing expression from a diseased *OPA1* allele capable of generating mutant *OPA1* protein would potentially aggravate disease progression, as more faulty *OPA1* protein would be present. In addition, all DNA-binding domains are capable of off-target binding (Cheng et al., 2011; Rogers et al., 2015; Zhang et al., 2015). Thus, in addition to the intended upregulation of *OPA1* expression, other genes might be impacted causing potential side effects. Also, in the context of DOA where precise control of *OPA1* expression is

necessary to avoid overproducing OPA1, fine tuning of ATF activity might prove difficult limiting therapeutic potential. Overall and despite these challenges, activation of gene expression with ATFs might prove useful for the treatment of a majority of DOA patients.

An alternative strategy to overcome haploinsufficiency through increase of OPA1 expression is to interfere with intrinsic mechanisms of its transcriptional or translational regulation. Alike a considerable fraction of protein coding genes in the human genome (Barbosa et al., 2013), the *OPA1* gene harbour an upstream open reading frame (uORF) in the 5' untranslated region (UTR) preceding the translational initiation site of the actual reading frame encoding the OPA1 protein. Such uORFs may act as negative regulators of translation since ribosomal occupancy of the uORF negatively impact 40S subunit scanning and recognition of the genuine *OPA1* start codon. In fact, ribosome profiling (Ingolia et al., 2009) shows a strong signal of ribosomal occupancy of the 5' UTR preceding the genuine start codon of both the human and the murine *OPA1* transcripts. In many studied examples, elimination of the uORF, e.g. through mutating of its AUG translation start codon, has been shown to reinforce translation of the actual ORF and elevate its protein level (Calvo et al., 2009; Chu et al., 2020; Suzuki et al., 2010). Therefore, means to suppress the translation of the uORF could be a valid strategy to target genetic disease due to haploinsufficiency. Antisense oligonucleotides (AONs) are synthetically manufactured short single-stranded oligonucleotides which bind to target sequences through base-pairing and thereby block access for native binding proteins or trigger enzymatic degradation (Bennett, 2019). In recent proof-of-concept studies, it was demonstrated that AONs which block the uORF or interfere with its secondary structure can increase translation efficiency and protein levels for a number of murine and human target genes including CFTR (Liang et al., 2016; Liang et al., 2017; Sasaki et al., 2019). It remains to be determined whether this effect can be generalized and more specifically holds true for the uORF of *OPA1* as a potential therapeutic approach for the fraction of patients with *OPA1*-linked disease, related to haploinsufficiency.

A majority of gene mutations are interpreted as deduced amino acid changes on protein level. However, it often is disregarded that mutations may firstly affect transcript processing and thus, have the potential to interfere with the splicing mechanism. Indeed, approximately 20% of all mutations cause splice defects, an observation that is almost independent from the mutated gene (Caminsky et al., 2014). This makes the splicing mechanism an attractive target for therapeutic approaches with a high potential to help a significant number of patients suffering from genetic diseases.

Therapies targeting the splicing machinery have been developed with the aim to correct the consequences of splice site mutations. In many cases, splice site mutations lower or abolish the affinity of splice factors resulting in mis-splicing of gene transcripts. Since a mutated splice site often leads to the activation of nearby cryptic splice sites, parts of transcripts may be skipped, elongated or truncated during the splicing mechanism (Cooper et al., 2009). Whole exons may be skipped due to single base

substitutions. To therapeutically correct fatal consequences of splice mutations, techniques that use modified splice factors are particularly promising. The best studied example of a modified splice factor is the small nuclear ribonucleoprotein U1 (U1). It has been shown that U1 is involved in the first steps of the splicing mechanism (Ast, 2004). With its RNA component, U1 binds to the splice donor site by complementary base pairing, subsequently recruiting complexes of other splice factors. Splice donor site mutations lower the affinity of U1 to its target, but the binding sequence of U1 can be adapted (U1^{adapt}) to match the mutated splice donor site and increase the affinity of U1^{adapt} to the target sites (Balestra et al., 2014; Da Costa et al., 2015; Dal Mas et al., 2015; Glaus et al., 2011; Nizzardo et al., 2015; Schmid et al., 2011; Schmid et al., 2013; Tanner et al., 2009). Consequently, U1^{adapt} initiates the splice process, recruits essential splice factors to the mutated splice donor site and facilitates regular splicing despite of the mutation. The application of the therapeutic U1^{adapt} has been shown to lead to increased levels of correctly splice transcripts in patient-derived cell lines and murine disease models (Balestra et al., 2014; Da Costa et al., 2015; Schmid et al., 2011).

For a successful treatment of some *OPAI* mutations affecting splicing, a treatment with U1^{adapt} would increase the level of correctly spliced *OPAI* transcript from the mutated allele without altering the delicate *OPAI* isoform ratios, thus treating the haploinsufficiency caused by the mutation. As a vehicle to apply the U1^{adapt} treatment to RGCs, AAVs seem to be the optimal choice and can easily incorporate the U1^{adapt} expression cassette, which size is below 1000 bp. The expression cassette includes its own promotor and consequently, obviates the need to optimize regulatory sequences to reach physiological expression levels. U1^{adapt} have been shown to efficiently treat mis-splicing for almost any splice donor site mutation. Despite of all these promising achievements towards a successful U1^{adapt} therapy, difficulties await developments before an application in patients may be considered. These include the more detailed pre-clinical testing of the therapeutic approaches in appropriate animal models. Furthermore, the U1^{adapt} therapy requires optimizations and modifications specific to the mutated splice donor site. Although obvious side effects have not been reported in patient-derived cell lines (Da Costa et al., 2015; Schmid et al., 2011), additional studies are required and it cannot be excluded that the U1^{adapt} interferes with the splicing of off-target transcripts in humans RGCs.

Besides mutations affecting the canonical splice sites and conserved residues of the splice donor and splice acceptor sequences, there is a growing number of newly identified deep-intronic mutations which impair proper transcript splicing and frequently result in the ‘activation’ and inclusion of pseudoexons in the mature RNA transcript. Such deleterious mutations have been described in *OPAI* in patients with DOA and Behr syndrome (Bonifert et al., 2014). Recognition and inclusion of such pseudoexons into the mature transcript can be efficiently suppressed through blockage of the splice donor or acceptor site with AONs including some *OPAI* mutations (Bonifert et al., 2016; Dulla et al., 2018; Sangermano et al., 2019). While there are a number of ongoing clinical trials with such splice-blocking or splice-switching AONs (Kuijper et al., 2020), and even a first approval for Spinraza® treatment for spinal

muscular atrophy, this strategy requires regularly re-administration to maintain a therapeutic level of the AON. Genome editing approaches may circumvent this re-administration and the dose monitoring through a permanent elimination of the defect at the level of the genomic DNA in somatic cells. While approaches relying on precise homology-directed repair in genome editing are still notoriously inefficient, the alternative strategy of a targeted double-strand breaks and repair by non-homologous end joining is much more efficient but at the expense of precision. However, small insertions or deletions usually observed in editing approaches are mostly tolerable in intronic sequences, but at the same time eventually destroy the splice donor or splice acceptor site of the pseudoexon, and thereby restore normal splicing of the mutant transcripts. Recently a successful Cas9-nickase approach was used to eliminate a frequent deep-intronic mutation in *CEP290* *in vitro* and *in vivo* in murine photoreceptors, upon subretinal delivery of CRISPR/Cas9 components applying AAV vectors (Maeder et al., 2019). This approach has now been put forward into clinical trials to treat patients with Leber Congenital Amaurosis type 10 (NCT03872479). Thus, this concept aiming at the elimination of deep-intronic mutations, using genome editing tools may well be applicable for other disease genes, including *OPA1*.

Lastly, an approach of trans-splicing of the endogenous *OPA1* pre-mRNAs could offer the opportunity to correct *OPA1* mutations at the transcript level. The process is based on enforcing the splicing machinery to branch the endogenous pre-mRNAs to a wild-type transgenic partial mRNA to eliminate the C-terminus sequence where is located the mutation (Koller et al., 2014). This trans-splicing technic would allow the correction of all alleles carrying a mutation downstream of exon 5b, thus respecting the normal expression pattern of all *OPA1* isoforms. Notably, this strategy could theoretically correct most of the haplo-insufficient and dominant-negative variants, and would not result in *OPA1* over-expression, as the trans-splicing process uses the endogenous *OPA1* pre-mRNA to generate a mature RNA devoid of the mutation (Liemberger et al., 2018).

Which of these alternatives that our teams are challenging will be efficient in pre-clinical trials performed with the different mouse models remains an open challenge, but should most probably lead to the perspective of clinical trials in the coming years.

7.2. Pharmacological and micro-nutritional protection of the optic nerve

Many pharmacological treatments for mitochondrial diseases have been proposed and challenged on different mouse models recapitulating human genetic pathologies. In most cases, they are based on the systemic administration of anti-oxidants drugs to counteract the abnormal ROS production induced by mitochondrial dysfunction, or of molecules like AICAR and Rapamycin, which promote the AMPK and mTOR pathways to activate mitochondrial respiration, and accelerate mitochondrial turn-over, in order to eliminate the less fit ones and produce more efficient ones. Alternatively, neuroprotective and growth factor treatments, based on the administration of proteins targeting survival pathways, like Nerve Growth Factor and Brain-derived Neurotrophic Factor, were envisioned to protect durably RGCs,

eventually through the implantation of a device into the eye vitreous allowing for a slow release of the active molecule. However, none of these possibilities has shown significant chronic protection of the optic nerve, and none was yet tested on *OPA1* mouse models to stimulate optic nerve survival.

Conversely, the use of Idebenone or EPI-743, which are synthetic analogues of coenzyme-Q10, has gained increasing interest in the last years as a possible treatment for inherited optic neuropathies. Indeed, Idebenone is now recognized in some European countries as a treatment for LHON during the first years after the acute loss of vision. Idebenone was also recently tested in an open trial on a cohort of DOA patients, leading to encouraging results reflecting a stabilization of the visual parameters and even a faint restoration of the visual acuity (Romagnoli et al., 2020). This could be either associated to the anti-oxidant effect of the molecule or to its capacity to stimulate the respiratory chain activity. Nevertheless caution should be considered when using Idebenone, as a randomized placebo-controlled trial of this molecule on the *Opa1*^{+STOP} mouse led to controversial data, disclosing some deleterious effects in aging wild-type animals, and ending to the conclusion that Idebenone is ineffective in protecting *Opa1*^{+STOP} RGCs (Smith et al., 2016). Thus, further pre-clinical and clinical studies are required to reach significant data concerning the benefit of Idebenone to treat DOA. Eventually further pharmacological studies should address the benefit of a targeted delivery of Idebenone into the eye vitreous, close to the RGCs, as this drug has unfavourable physicochemical properties leading to poor bio-availability, which compromise its administration by oral and topical medications (Montenegro et al., 2018).

An opportunistic and intuitive alternative to drug treatments, would rely on micro-nutrients' supplementation of DOA patients. Because *OPA1* metabolomics signature evidenced deficiencies in many compounds, among which some are essential, as amino-acids and vitamins, one could anticipate that the replenishment of the pools of these compounds either by specific diets or by micro-nutrients supplementation could somehow restore mitochondrial and cellular physiology, up to a level sufficient to slowing down the evolution of the disease. In particular, this could apply early during the life time, well before the major loss of RGCs and visual activity. Two compounds which were depleted in the plasma signature of *OPA1* patients are gaining attention: one is taurine, a secondary amine that already showed promising effects for RGC neuroprotection (Froger et al., 2014), and the other one is nicotinamide, a precursor of vitamin B3, which administration at high concentration virtually stops RGC degeneration in the DBA-2J mouse model of glaucoma (Williams et al., 2017). This latter effect is related to the anti-oxidant property of the vitamin B3, or to its contribution to the synthesis of NAD⁺, which fuels the complex I from the respiratory chain. Notably, in plasma from glaucoma and *OPA1* patients, a 33% decrease in nicotinamide was found (Bocca et al., 2018b; Kouassi Nzoughe et al., 2019), although at different ages. This supports a convergence of their respective pathomechanisms to a mitochondrial complex I dysfunction, reflecting a premature RGC aging that could be restrained by vitamin B3 supplementation. This latter hypothesis deserves a future double blind clinical trials on a

large cohort of *OPA1* patients, in order to assess the neuroprotective activity of nicotinamide on the vision of DOA patients. Thus, these available opportunities for pharmacological and nutritional approaches could be considered now to stabilize their visual capacities, while expecting a gene therapy solution to be efficient in the next years, allowing a “one shot” acute and definitive treatment of *OPA1* patients.

7.3. The ultimate goal: RGC and optic nerve regeneration

While the therapeutic approaches described in the former paragraphs intent to slow down and halt the RGC degeneration, the utmost perspective to treat DOA and other optic neuropathies would be the restauration of a normal optic nerve by RGC regeneration. Two recent papers are supporting the feasibility of this perspective. The first one proved that mouse Muller glial cells can be converted efficiently to RGCs by targeted downregulation of *Ptbp1*, a gene encoding the RNA-poly-pyrimidine tract-binding protein 1. This molecular manipulation led to functional RGCs able to alleviate the symptoms of an optic neuropathy, emphasizing the potential of glia-to-neuron conversion for restoring RGCs in DOA patients (Zhou et al., 2020). In addition, grafting Schwann cells in vitreous was shown to promote the secretion of neurotrophic factors and RGC axon regeneration through the optic nerve, despite the presence of inhibitory molecules that prevent axon growth in anastomosis situations (Ahmed et al., 2020). Thus, these preliminary encouraging results open perspectives to restore a functional optic nerve and improve visual acuity of patients with an optic neuropathy, and are futuristic objectives to consider for treating the premature loss of sight in a significant proportion of DOA patients.

Legends to the figures

Figure 1. Ophthalmological findings in a 26-year-old man with a confirmed pathogenic OPA1 mutation (c.1057A>C, p.Ser353Arg).

The best-corrected visual acuity was 20/30 in his right eye and 20/40 in his left eye. (A) Fundus photography showing temporal pallor of the optic nerve in both eyes. (B) Bilateral small central scotomas were present on Humphrey visual field perimetry. (C) OCT imaging confirmed thinning of peripapillary RNFL that was more marked in the temporal quadrant corresponding to the papillomacular bundle.

Figure 2. Genes causing DOA and their associated phenotypes.

Synthesis of all DOA genes identified. *OPA1* was originally described in families with isolated optic atrophy, whereas all other genes causing DOA were identified first in patients with multisystemic disease. In addition, bi-allelic mutations in DOA have been described in patients presenting with syndromic optic atrophy (Behr syndrome) and other severe syndromes involving encephalopathy +/- cardiomyopathy.

Figure 3. Consequences of OPA1 dysfunction on the IMM and OMM shapes.

Electron microscopy of a cell depleted for OPA1 (*OPA1*^{-/-}) reveals a major disruption of the mitochondrial cristae structure (left) compared with the control condition (*OPA1*^{+/+}) showing normal cristae structures (center top). Fluorescence microscopy of a cell depleted for OPA1 reveals a highly fragmented mitochondrial network (right), whilst the control condition shows tubular and elongated mitochondria (center bottom). OPA1 regulated further intra (left) and inter (right) mitochondrial functions related to respiration, mtDNA maintenance, apoptosis and calcium metabolism.

Figure 4. Assessment of mitochondrial abundance in mouse retinal ganglion cells and the optic nerve.

Mitochondria were labelled using a primary antibody against OPA1 (green). The optic nerve structures were labelled (red) with an antibody directed against MBP to localize the myelin, or with an antibody against the neurofilament NF200 to detect the axons. (A) Retinal section with the emerging optic nerve showing a high abundance of mitochondria, (B) Mitochondria are more abundant in the unmyelinated segment of the optic nerve (MBP labeling is absent) and there is marked reduction with the presence of myelin (intense MBP labeling). (C & C') and (D & D') Mitochondrial abundance remains low from the optic chiasm to the optic tracts compared with the surrounding tissues. (E & E') Mitochondrial labeling at the lateral geniculate nucleus, where are located the RGC synapses.

Figure 5: Electron microscopy showing the changes in density and morphology of mitochondria along the optic nerve in relation to myelination.

Mitochondria are abundant in the unmyelinated segment of the optic nerve (left panel), with a round shape and clear matricial content. In contrast, mitochondria are scarce in the myelinated segment (right

panel), with a tubular shape and a dark matricial content. (middle) At the transition region between these two segments, mitochondria evolve from a round shape (arrow head) to an elongated shape (arrow), and their matricial content change from clear to dark, following the progression of axonal myelination.

Figure 6: Schematic representation of a retinal ganglion cell and its dual environmental conditions before and after the lamina cribrosa.

A retinal ganglion cell (RGC) is schematically represented with the soma at the left, the synapse on the right, and the axonal segment stretching in between. The red spots represent fragmented mitochondria in the unmyelinated region of the axon on the left part of the figure, whereas the large scarlet oval shapes represent mitochondria distributed in the myelinated part of the axon. The lamina cribrosa (LC) is the anatomical frontier separating these two different axonal environments, which are under different influences, namely intraocular pressure (IOP) and intracranial pressure (ICP), and energy requirements depending on myelination. The dynamic nature of the mitochondrial membranes is a key feature that allows rapid adaption of mitochondrial IMM and OMM shapes in both physiological and disease states, and this crucial process is regulated by OPA1.

Figure 7: Chronological comparison of the molecular and phenotype features associated with three *Opa1* mouse models.

The left hand panel lists the features that have been analyzed in each mouse model. For each of them, the red spots indicate the time points when significant differences were documented when compared with controls, whereas the green spots indicate that no significant difference was found. The three mutant heterozygous mouse models compared are: *Opa1*^{+/*STOP*}, *Opa1*^{+/*delTTAG*} and *Opa1*^{+/*del*}.

Figure 8: Synthetic metabolomic signatures associated with OPA1 dysfunction.

Five major pathological changes in metabolomic profile have been observed in both *Opa1* mouse models, and in fibroblast and plasma from individuals with DOA carrying known pathogenic *OPA1* mutations. These changes are represented with their possible consequences on mitochondrial and cell physiology. Changes in NAD metabolism, and aspartate and glutamate concentrations, reflect mitochondrial respiration chain deficiency. Increased syntheses of antioxidant compounds indicate possible ROS overproduction. The modifications of phospholipids biosynthesis are potentially related to adaptive changes membrane dynamics and cristae structure. Changes in purine and pyrimidine metabolisms could be causally related to the mtDNA instability related to the loss of OPA1. The alterations of sphingolipid and sphingomyelin metabolism point towards the disorganization and eventual destruction of the axonal myelin sheath.

Declaration of interests

All authors declare that they have no declaration of interest to disclose.

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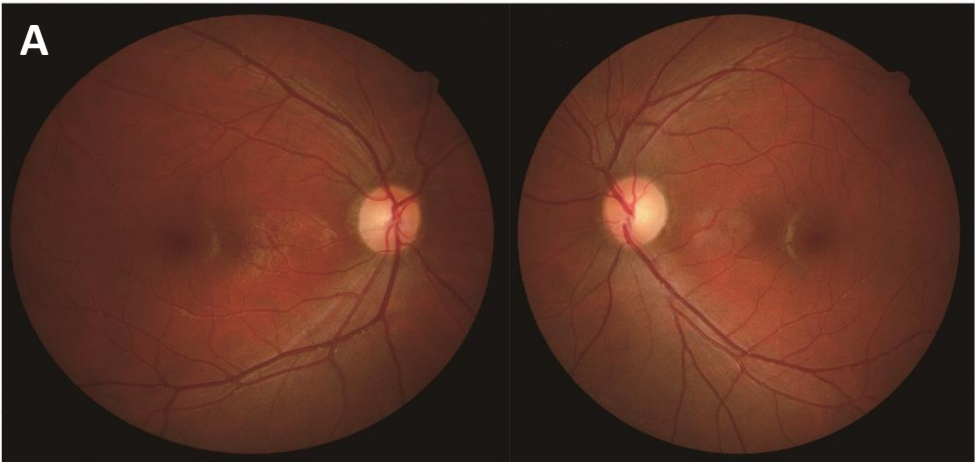
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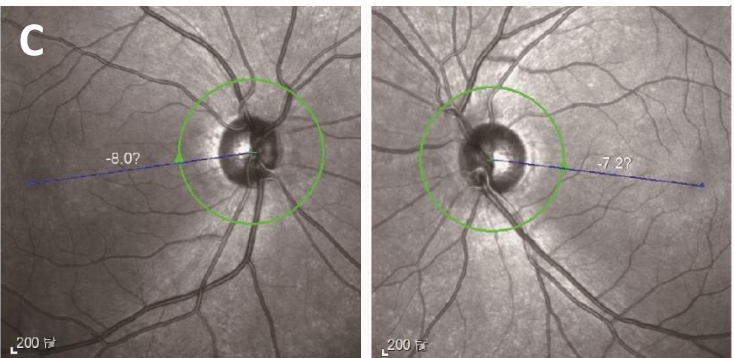
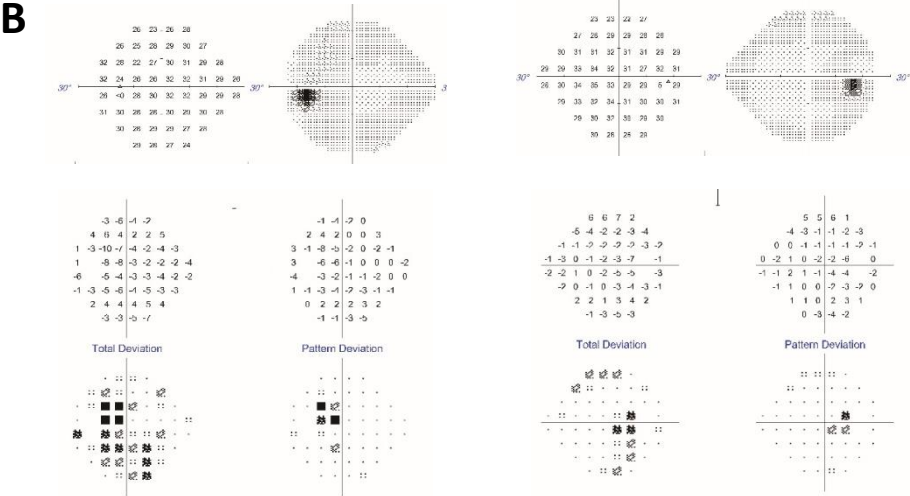
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Figure 1



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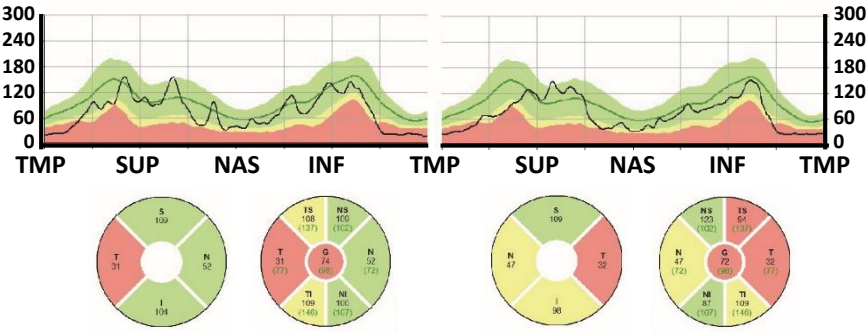
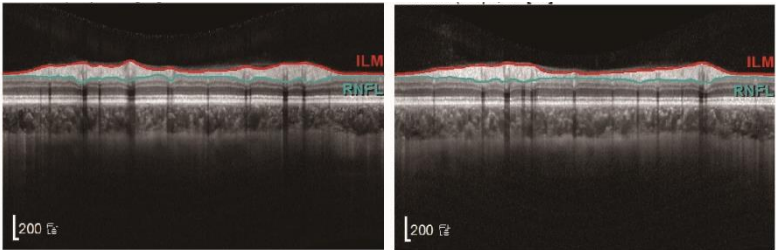


Figure 2

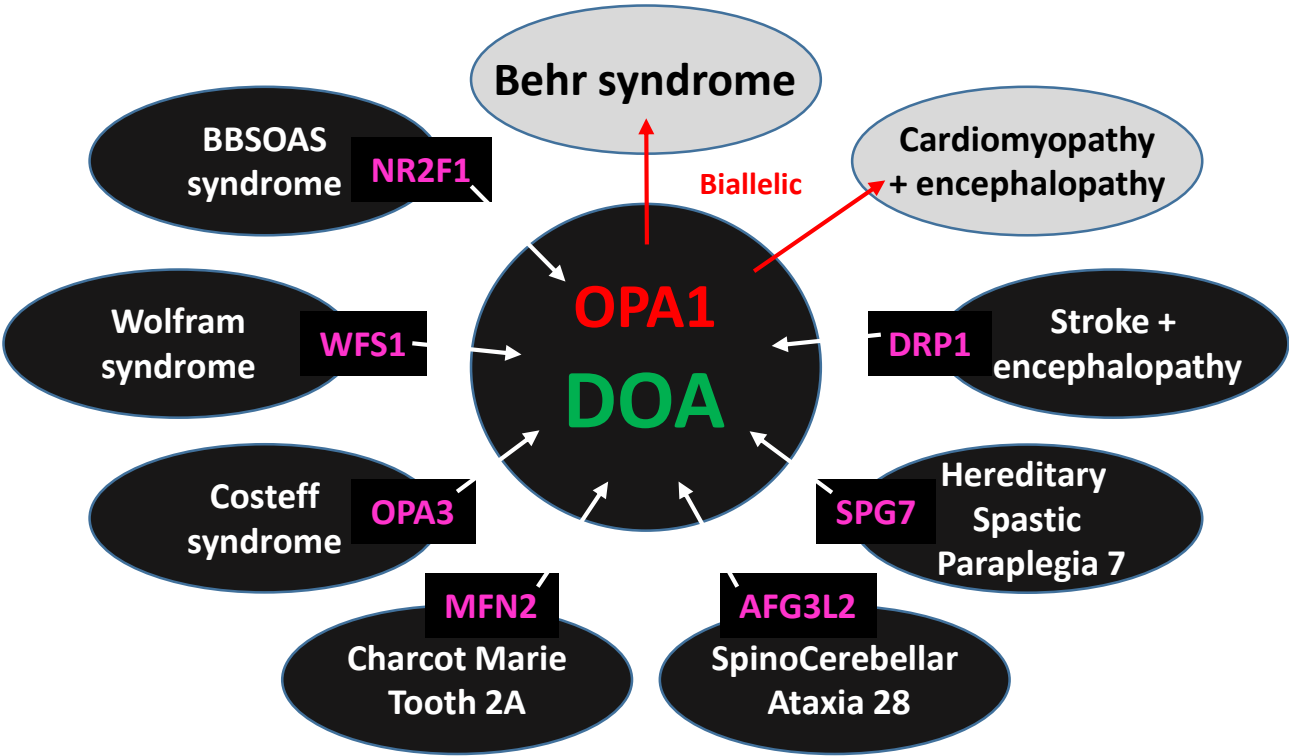


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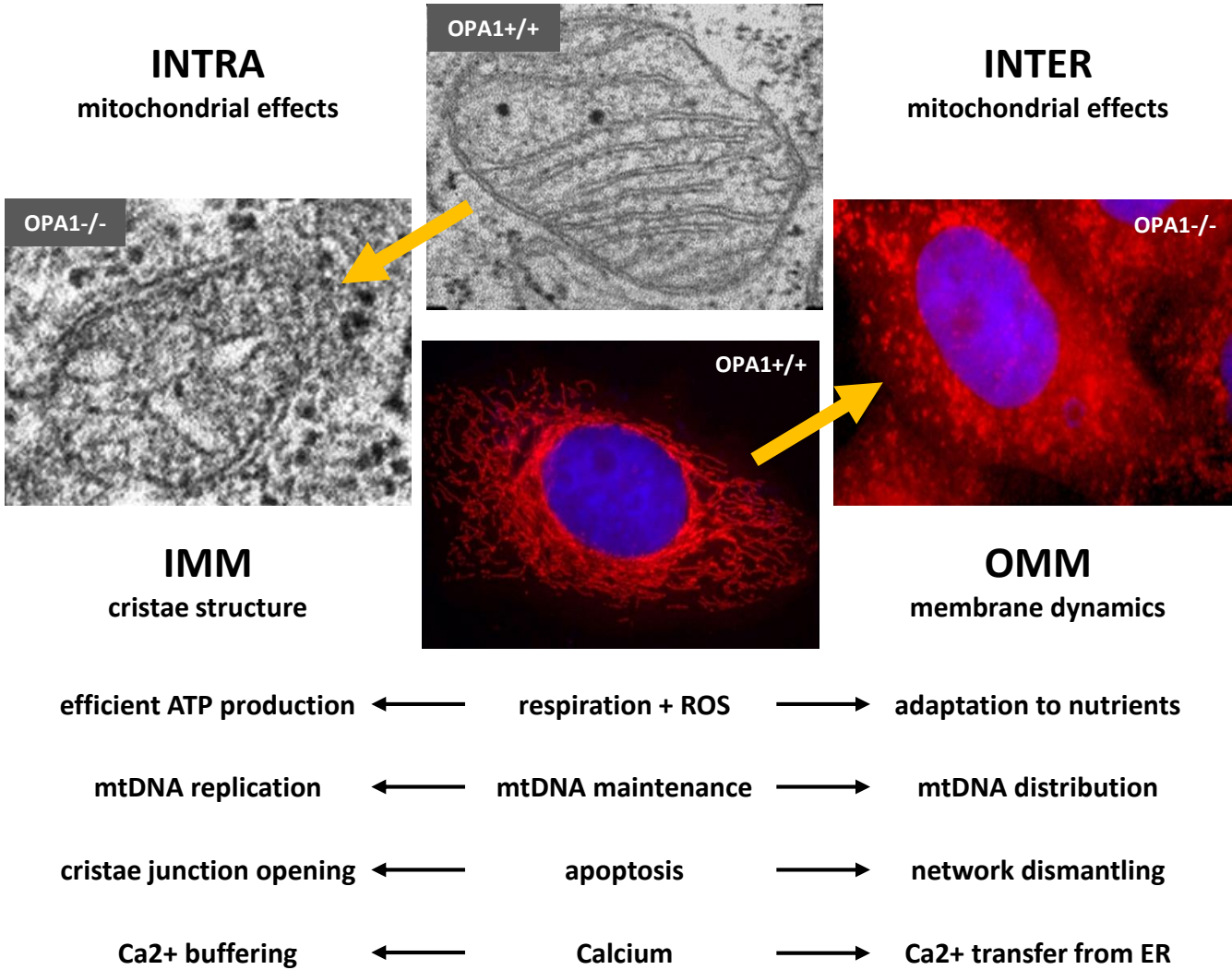


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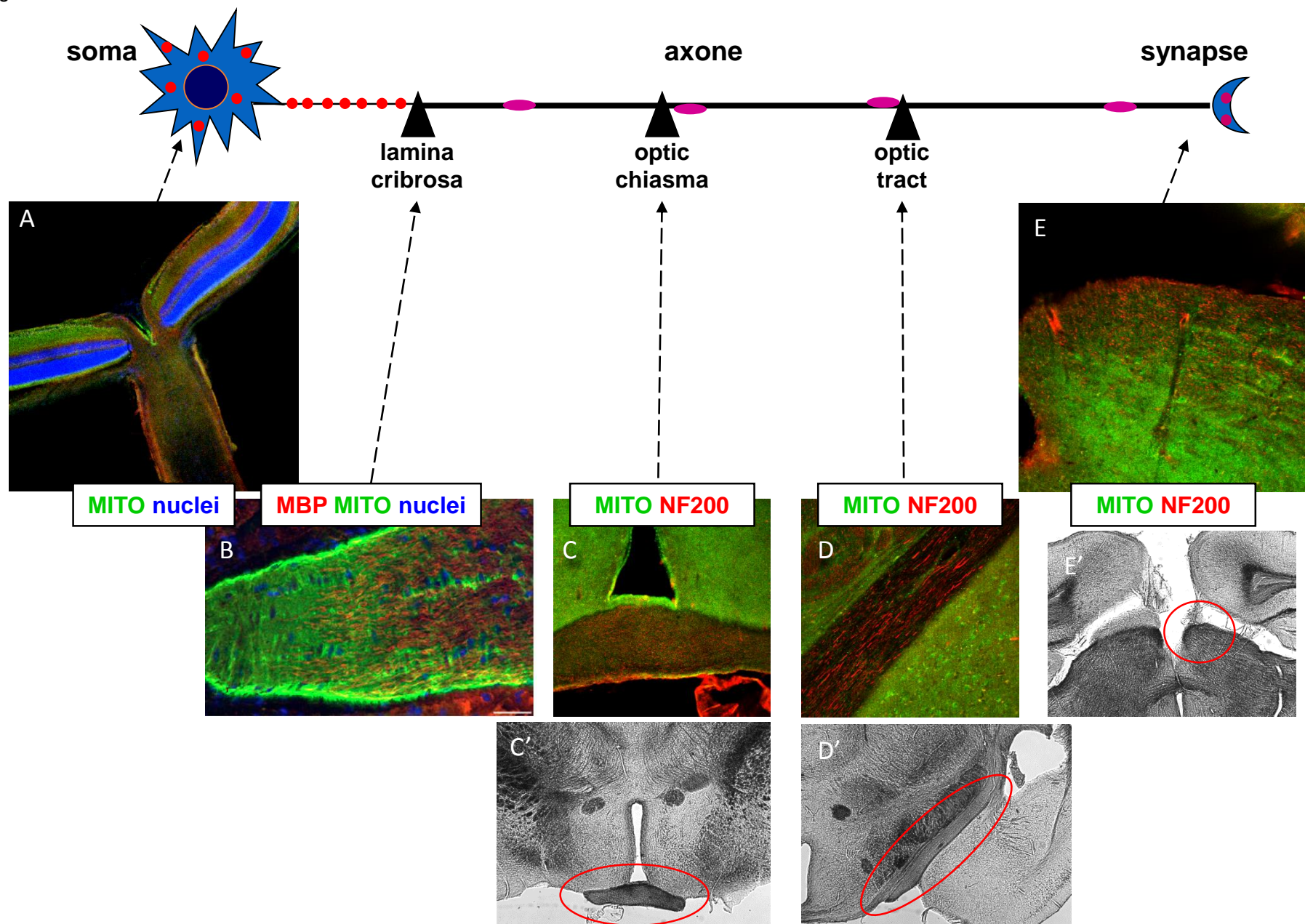


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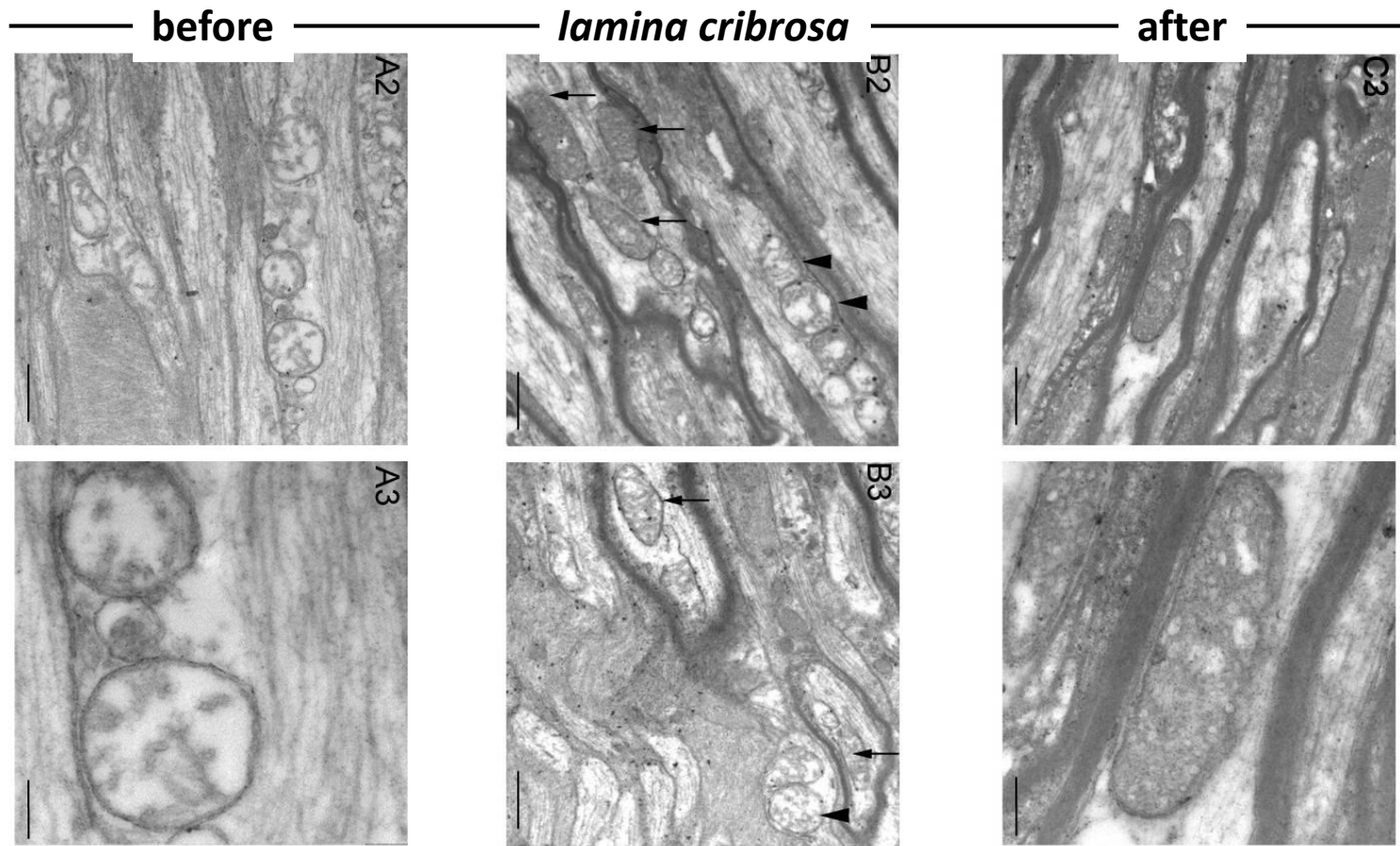


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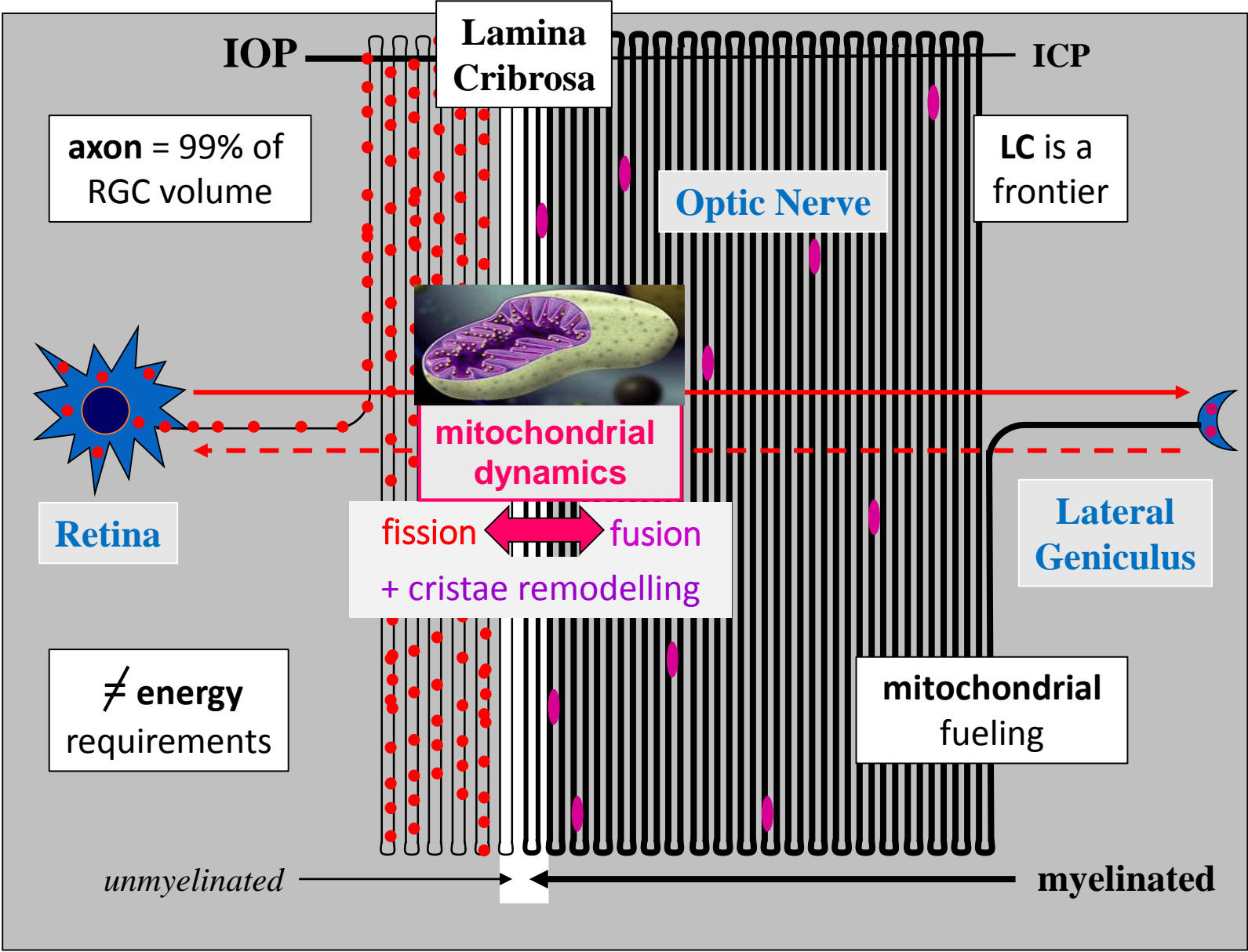


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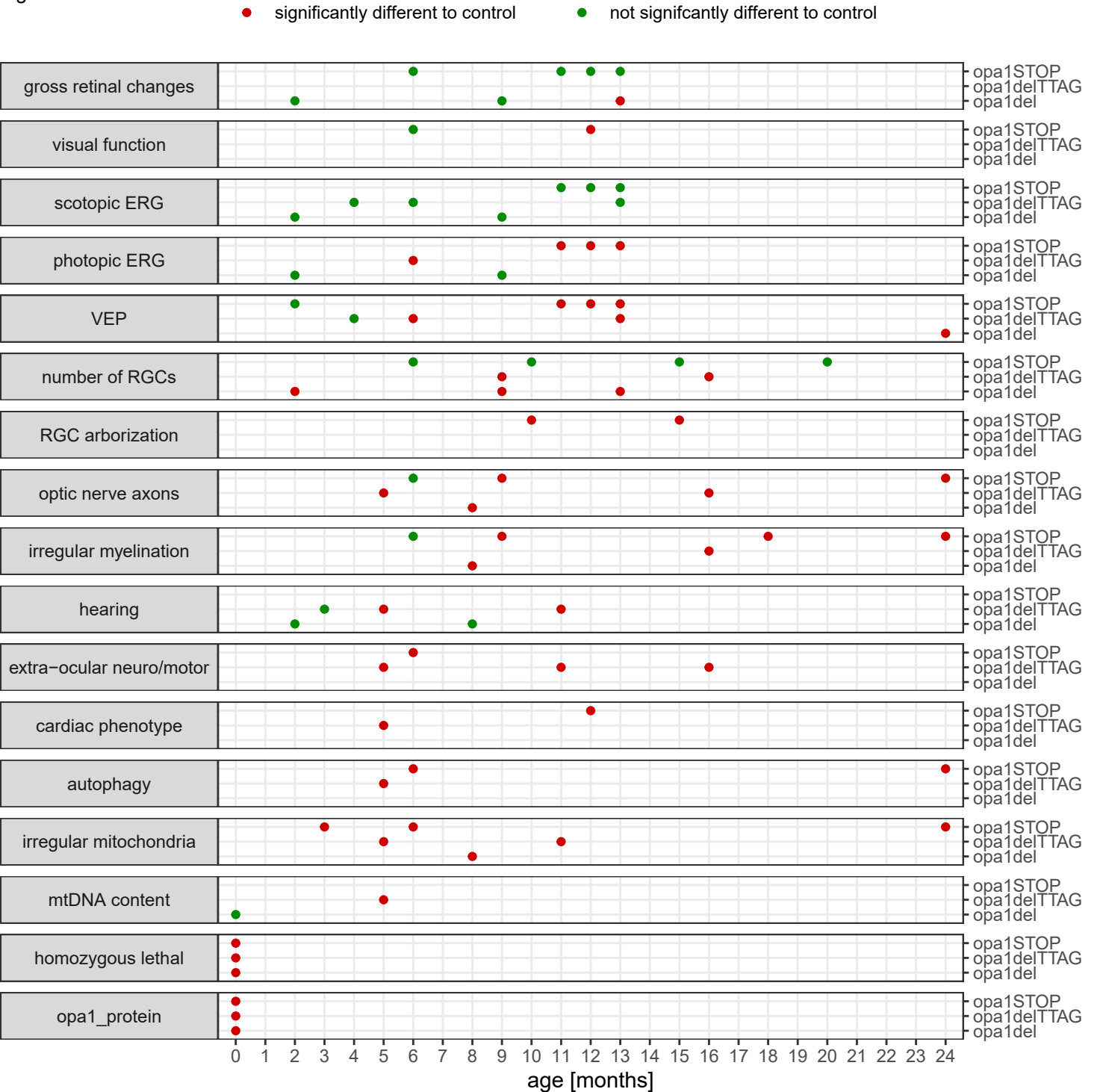
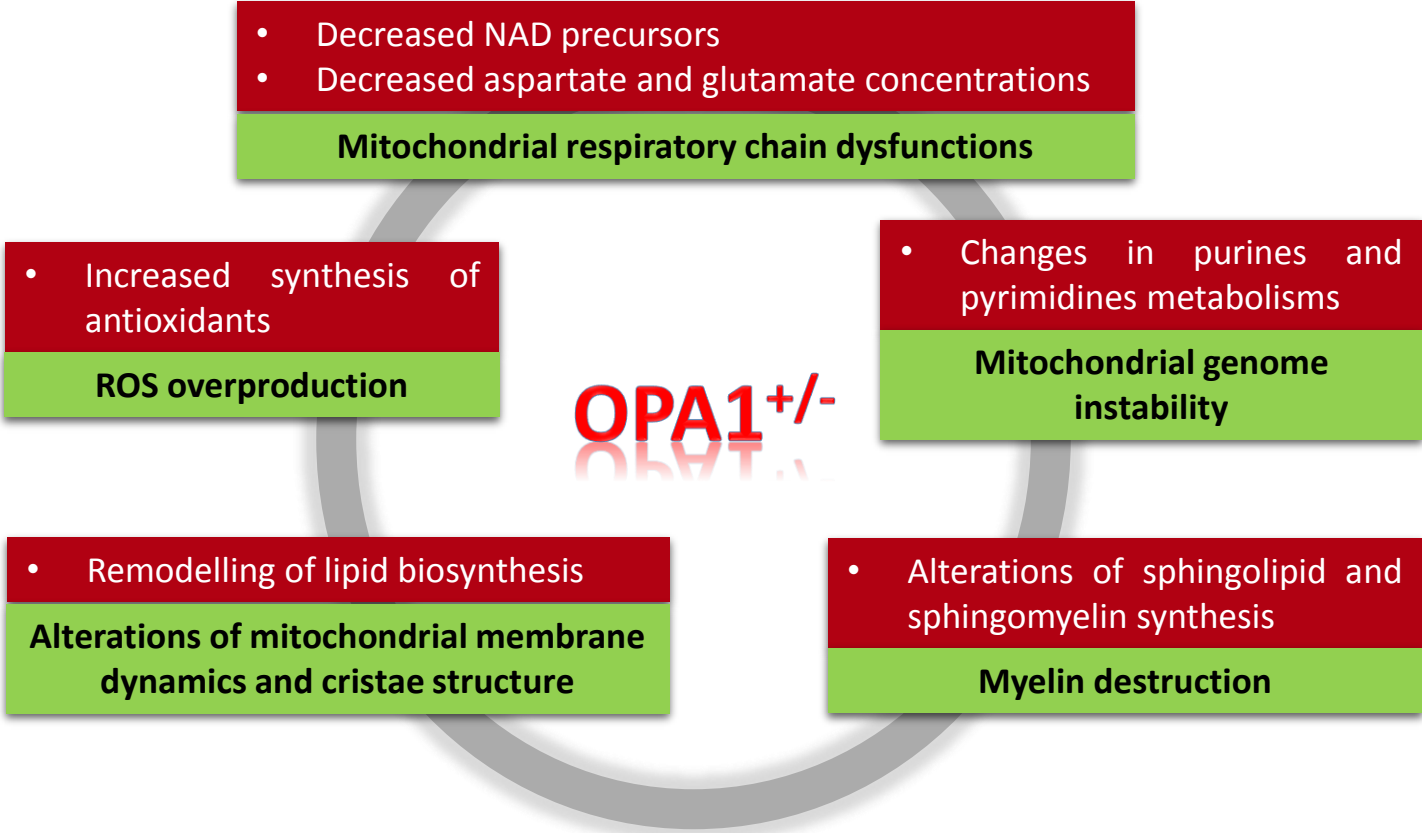


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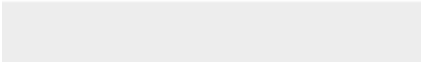




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Table

Table 1 DOA genes.docx



Dominant Optic Atrophy: culprit mitochondria in the optic nerve

Author statement.

Declaration of interest:

All authors certify that they have no declaration of interest to disclose.

Dominant Optic Atrophy: culprit mitochondria in the optic nerve

AFFILIATIONS

RELATIVE CONTRIBUTION OF EACH AUTHOR

KEYWORDS

LIST OF ABBREVIATIONS

ABSTRACT

MAIN BODY OF MANUSCRIPT

1. DOA IN CLINICS
 - 1.1. *Common clinical features of DOA*
 - 1.2. *Expanding DOA genotypes and phenotypes*
2. GENETICS AND RISK FACTORS
 - 2.1. *Mutations in OPA1*
 - 2.2. *OPA1 Phenotype-genotype correlations*
 - 2.3. *Additional DOA loci and genes*
 - 2.4. *Genetic diagnostics*
3. THE CULPRIT MITOCHONDRIA
 - 3.1. *OPA1 structure and expression*
 - 3.2. *OPA1: a mitochondrial “Swiss army-knife”*
 - 3.3. *OPA1 and cristae structure and remodeling*
 - 3.4. *OPA1 in mitochondrial fusion*
 - 3.5. *OPA1 and the control of energy metabolism*
 - 3.6. *OPA1 in the regulation of mitochondrial calcium uptake*
 - 3.7. *OPA1 and the control of apoptosis*
 - 3.8. *OPA1 and the maintenance of the mitochondrial genome*
 - 3.9. *Alternative extra-mitochondrial OPA1 functions*
 - 3.10. *Functions of the other genes involved in DOA*
4. THE PATHOPHYSIOLOGY OF DOA
 - 4.1. *Why RGCs and the optic nerve degenerate in DOA?*
 - 4.2. *Why specifically the RGCs from the papillo-macular bundle degenerate in DOA?*
5. ANIMAL MODELS OF DOA
 - 5.1. *Mouse models of DOA*
 - 5.2. *Characterization of the $Opa1^{+/del}$ mouse model of DOA*
 - 5.3. *Expression of DOA phenotypes in the $Opa1^{+/STOP}$ mouse model*
 - 5.4. *The $Opa1^{+/delITAG}$ model of DOA*
 - 5.5. *Modeling DOA in other organisms*
6. METABOLOMICS CONSIDERATIONS IN DOA
 - 6.1. *Qualitative aspects*
 - 6.2. *Quantitative aspects*
7. CONCLUSIONS AND FUTURE DIRECTIONS TOWARDS THERAPIES
 - 7.1. *Gene therapy for DOA*
 - 7.2. *Pharmacological and micro-nutritional protection of the optic nerve*
 - 7.3. *The ultimate goal: RGC and optic nerve regeneration*

LEGENDS TO THE FIGURES

Figure 1. Ophthalmological findings in a 26-year-old man with a confirmed pathogenic OPA1 mutation (c.1057A>C, p.Ser353Arg).

Figure 2. Genes causing DOA and their associated phenotypes.

Figure 3. Consequences of OPA1 dysfunction on the IMM and OMM shapes.

Figure 4. Assessment of mitochondrial abundance in mouse retinal ganglion cells and the optic nerve.

Figure 5: Electron microscopy showing the changes in density and morphology of mitochondria along the optic nerve in relation to myelination.

Figure 6: Schematic representation of a retinal ganglion cell and its dual environmental conditions before and after the lamina cribrosa.

Figure 7: Chronological comparison of the molecular and phenotype features associated with three *Opa1* mouse models.

Figure 8: Synthetic metabolomic signatures associated with OPA1 dysfunction.

DECLARATION OF INTERESTS

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REFERENCES

Dominant Optic Atrophy: culprit mitochondria in the optic nerve

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