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# Eight human OPA1 isoforms, long and short: What are they for?

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## ABSTRACT

OPA1 is a dynamin-related GTPase that controls mitochondrial dynamics, *cristae* integrity, energetics and mtDNA maintenance. The exceptional complexity of this protein is determined by the presence, in humans, of eight different isoforms that, in turn, are proteolytically cleaved into combinations of membrane-anchored long forms and soluble short forms. Recent advances highlight how each OPA1 isoform is able to fulfill “essential” mitochondrial functions, whereas only some variants carry out “specialized” features. Long forms determine fusion, long or short forms alone build *cristae*, whereas long and short forms together tune mitochondrial morphology. These findings offer novel challenging therapeutic potential to gene therapy.

### Keywords:

OPA1

Long and short OPA1 forms

Mitochondrial network dynamics

*Cristae*

mtDNA

Energetics

## 1. Introduction

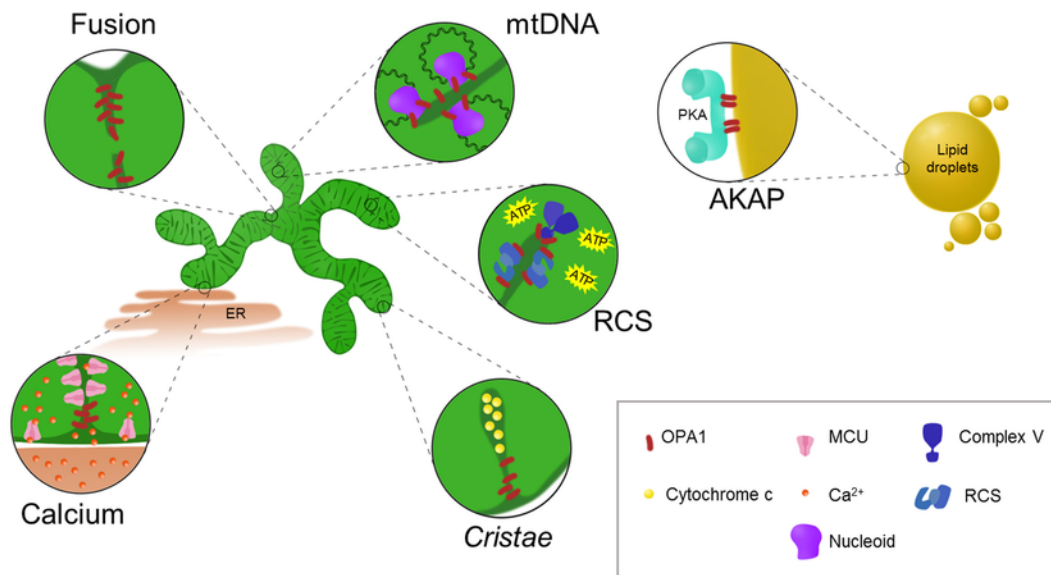
The mitochondrial network morphology is the result of a constant dynamic balance between organelles fusion and fission, adapting the cell to the energetic requests and preserving the homeostasis and the quality of mitochondria. The protein machinery deputed to mitochondrial dynamics includes Mitofusins (MFN1 and MFN2) and DRP1, respectively involved in fusion and fission of the outer mitochondrial membrane (OMM), and the conserved dynamin-like GTPase OPA1 that plays a crucial role in the inner mitochondrial membrane (IMM) fusion [1].

Growing evidence indicates that, in addition to fusion, OPA1 is implicated also in other important mitochondrial functions, such as mitochondrial DNA (mtDNA) maintenance, probably by anchoring this genome to the IMM [2], the respiratory chain supercomplexes (RCS) assembly and the energetic efficiency [3]. OPA1 is also required for *cristae* structure organization [3] and apoptosis regulation through the compartmentalization of soluble cytochrome *c* within the *cristae* [4,5]. Recently, OPA1 has been shown to promote and regulate the mitochondrial pH flashes, which are bioenergetic responses to drops in mitochondrial membrane potential ( $\Delta\psi_m$ ) [6,7]. OPA1 also plays a significant role in the control of mitochondrial  $\text{Ca}^{2+}$  homeostasis [8] (Fig. 1).

In the year 2000, OPA1 mutations were for the first time reported as causative for dominant optic atrophy (DOA), a blinding disease

characterized by selective degeneration of the retinal ganglion cells (RGCs) and optic nerve atrophy [9]. About 50% of the pathogenic mutations are predicted to produce a truncated protein, indicating haploinsufficiency as the molecular mechanism causing DOA. The missense mutations, frequently clustered in the GTPase domain and assumed to exert a dominant-negative effect, are in most cases associated with the severe multisystem disorder recognized as DOA “plus”. This is characterized by a multi-systemic involvement, associated with a large spectrum of clinical features, including Parkinsonism and dementia, as well as a disorder indistinguishable from multiple sclerosis [10].

The investigation of humans and mouse models identified further, more tissue-specific functional roles of OPA1. Retinal studies of an Opa1 haploinsufficient mouse model showed that the expression of the glutamate NMDA receptors was significantly increased [11] and OPA1 depleted RGCs were more susceptible to glutamate excitotoxicity [12]. OPA1 also seems to be somehow linked with aging, in fact selective loss of glutamatergic, but not GABAergic, synaptic sites, leading to dendritic degeneration was reported in aged Opa1 +/– mice [13]. Sedentary but not active humans display an age-related decline of OPA1 protein levels associated with muscle loss [14] and OPA1, together with MFN1, regulates the metabolic shift from glycolysis to mitochondrial respiration in old human fibroblasts during chronological lifespan [15]. In adipocytes cellular triacylglycerol accumulation is regulated, at least in part, via mitochondrial fusion and fission processes, with mitochondrial morphology altered from filamentous to fragmented upon differ



**Fig. 1.** Mitochondrial and extra-mitochondrial OPA1 functions. Schematic representation of OPA1 functions. OPA1 is responsible for IMM fusion and, as a component of nucleoids, is involved in mtDNA maintenance by its anchoring to the IMM. It is crucial for the proper assembly of respiratory chain supercomplexes (RCS) and Complex V. OPA1 participates to the *cristae* organization, keeping tight the *cristae* junctions, and to apoptosis, by cytochrome *c* compartmentalization. OPA1 is implicated also in mitochondrial calcium homeostasis at the mitochondrial associated membranes (MAMs), endoplasmic reticulum (ER) subdomains in close contact with mitochondria. In adipocytes OPA1 acts, on lipid droplets surface as A-kinase anchoring protein (AKAP), thus regulating the lipid metabolism. MCU (mitochondrial calcium uniporter).

entiation to adult adipocytes [16]. Furthermore, OPA1 has been proposed to anchor the A-kinase to lipid droplets to mediate the adrenergic control of lipolysis [17] (Fig. 1). Consistently perturbation of OPA1 processing causes obesity and defective thermogenesis in mice [18].

All together these findings support a multiplicity of cellular functions for this dynamin related GTPase, in most cases strictly associated with the classical mitochondrial location but a few possibly occurring also in extra-mitochondrial compartments. Further studies are needed to better understand the relevance of these latter aspects within mitochondrial functions.

## 2. OPA1 protein: Structure and isoforms

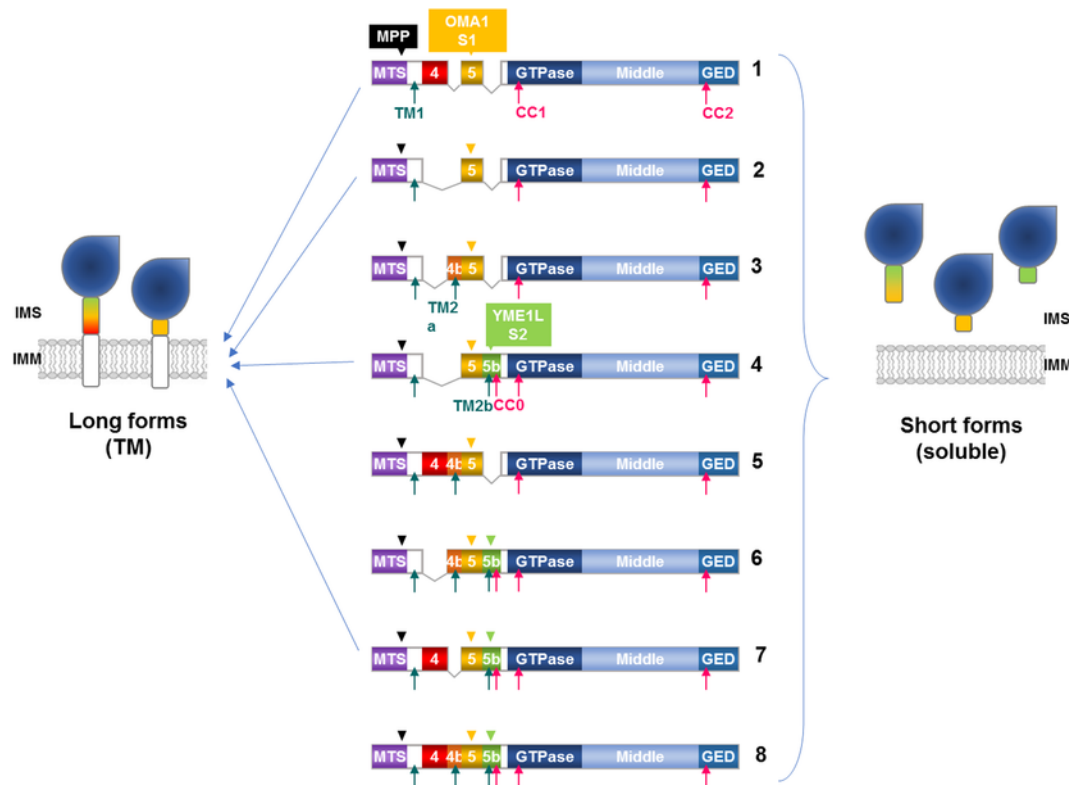
The OPA1 protein is localized in the mitochondrial intermembrane space (IMS), anchored to the IMM. In humans, OPA1 is present in eight mRNA variants, deriving from the alternative splicing of exons 4, 4b and 5b, encoding proteins of 924–1014 aminoacids, whose N-terminus includes a mitochondria targeting sequence, followed by a transmembrane domain (TM), embedded in the IMM, and a coiled coil domain. The next portion of the protein includes three highly conserved dynamin regions: the GTPase domain, the middle domain and the C-terminus GTPase effector domain (GED) [19] (Fig. 2). The eight different isoforms are ubiquitously expressed but present in different amount depending on the tissue considered [20]. After import of the precursor protein through the OMM and IMM translocases, cleavage of the mitochondrial targeting sequence generates the membrane-anchored OPA1 long forms (l-forms) that may be further proteolytically processed at the N terminus producing the short forms (s-forms) soluble in the IMS [21]. The four isoforms containing the exon 4b are totally processed into the soluble s-forms [22]. Two IMM peptidases are involved in the process, OMA1 operating at the cleavage site S1 in exon 5, and YME1L at site S2 in exon 5b (Fig. 2). Under normal conditions, YME1L is constitutively active, whereas OMA1 is inactive, but can be activated by stress conditions and mitochondrial dysfunction (dissipation of  $\Delta\Psi_m$ ) [23,24], increasing the s-forms. Activated OMA1 was shown to undergo autocatalytic self-degradation, thus allowing for mitochondrial network recovery [23]. Interestingly, during mitochondrial depolarization the two proteases are differentially degraded through a mechanism

controlled by cellular ATP availability. In fact treatments that drop both  $\Delta\Psi_m$  and ATP levels stabilize active OMA1 and provoke YME1L degradation, strongly influencing OPA1 processing and the pattern of the s-forms [25]. The complex reciprocal degradation of the two proteases can therefore profoundly affect the balance of l-/s-forms as well as the network morphology.

The unbalance toward the OPA1 s-forms, fusion inhibition and unopposed network fragmentation have fundamental consequences within the cell, triggering the activation of a process referred to as “mitochondrial quality control” or MQC. This marks the isolated mitochondrial fragments with reduced  $\Delta\Psi_m$  toward their removal by autophagy, a process defined as mitophagy [26,27]. Signaling of and marking dysfunctional mitochondria for mitophagy is driven by a complex machinery of factors, which includes the PINK1/Parkin axis, a hot topic for the pathogenesis of Parkinson disease [28]. Interestingly, the E3 ubiquitin ligase Parkin, through linear ubiquitination of NF- $\kappa$ B essential modulator (NEMO) may also regulate the expression of OPA1 [29]. On the contrary, mitochondrial network elongation, e.g. during starvation, was shown to hinder autophagic degradation [30]. There is therefore a tight homeostatic relationship between mitochondrial dynamics, energetic status and MQC. Remarkably, recent results reported in fibroblasts bearing different OPA1 mutations, including those linked to Parkinsonism, demonstrate excessive mitochondrial network fragmentation which results into increased mitophagy [31,32]. However, a detailed understating of the mechanistic interplay between mitochondrial dynamics and MQC needs further studies on a larger number of patients and by refining reliable quantitative mitophagy assays, under different metabolic conditions.

## 3. Role of the eight OPA1 isoforms in mitochondrial functions

A great variability of the eight OPA1 isoforms was detected in different human tissues, suggesting a fine regulation of alternative splicing of OPA1 mRNA. To gain insights on the phenotypes associated with the expression of different isoforms, selective silencing of each of the three alternative exons was performed in HeLa cells, where the variants bearing exon 4 are the most abundant. Noticeably, the mitochondrial morphology of exon 4 silenced cells exhibits highly fragmented net-



**Fig. 2.** OPA1 proteins structure and proteolytic processing. In humans, *OPA1* is alternatively spliced to generate eight variants (isoform 1–8). After import in the IMM, the mitochondrial targeting sequence (MTS) is cleaved by the mitochondrial processing peptidase (MPP) to generate the long TM forms. Transmembrane domains (TM1, TM2a, TM2b) and coiled coil domains (CC0, CC1, CC2) are indicated with blue and red arrows, respectively. All isoforms contain exon 5 with the S1 cleavage site (yellow arrowhead), whereas half of them (isoforms 4, 6, 7, 8) contain also the S2 cleavage site (green arrowhead). Proteolytic cleavage at S1 and S2 cleavage sites by the OMA1 and YME1L peptidases generates the IMS soluble short forms.

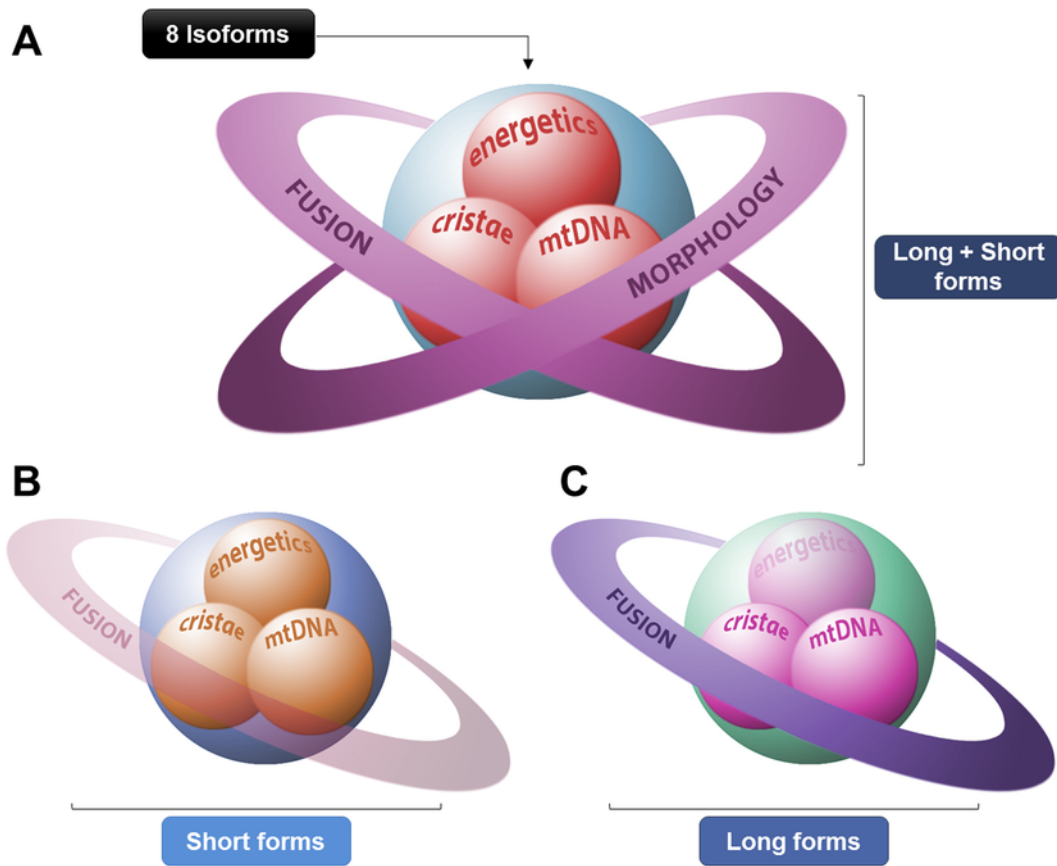
work and loss of  $\Delta\Psi_m$ , whereas both exon 4b and 5b silenced cells display typical hallmarks of apoptotic cell death [20]. Interestingly, only exon 4b silenced cells displayed mtDNA depletion and a marked alteration of nucleoids distribution throughout the network. Thus, the OPA1 N terminus, including exon 4b, may contribute to nucleoid attachment to the IMM, promoting and regulating mtDNA replication, nucleoids abundance and distribution [2]. These findings seem to indicate that different OPA1 isoforms may be associated with specific mitochondrial functions, with the hypothesis that the exon 4 containing variants could be involved in mitochondrial fusion, the exon 5b variants could keep tight the *cristae* junctions to prevent cytochrome *c* mobilization and the exon 4b variants could maintain the mtDNA.

Nevertheless, this latter experimental approach does not allow to evaluating whether a single OPA1 isoform is specifically associated with a definite mitochondrial function. To address this issue, a detailed molecular and biochemical analysis was carried out in *Opa1*-null cells where the individual *OPA1* splice forms have been stably expressed alone, demonstrating that expression of any OPA1 isoform is able to maintain the physiological level of mtDNA content, to restore the *cristae* structure, and to preserve the supra-molecular organization of respiratory complexes, as well as the fully assembled complex V, all these features being severely impaired in *Opa1*  $-/-$  MEFs [33]. The mitochondrial network morphology, completely fragmented in *Opa1*  $-/-$  MEFs, is partially rescued only by mRNA splice forms generating both l- and s-forms, in agreement with a previous report [22]. By using a combination of isoforms silencing and co-expression experiments, it emerges that the presence of at least two isoforms with a specific balance of l- and s-forms and an adequate amount of protein are

both required to fully recover the mitochondrial network morphology [33]. Thus, the multiplicity of OPA1 isoforms is possibly necessary to finely tune mitochondrial dynamics under different cellular conditions. This complex array of combined OPA1 isoforms most probably provides the necessary cellular flexibility to shape mitochondrial dynamics as adaptive response in different metabolic and stress conditions that may perturb mitochondrial homeostasis in highly specialized tissues.

Noteworthy, the analysis of cell models where the eight OPA1 isoforms are individually expressed, highlights the feature that each one has the potential to preserve three essential phenotypes, i.e. mtDNA content, energetics and *cristae* structure, acting as a “pluripotent” isoform, this term being reminiscent of the ability of stem cells to perform different specialized functions, undergoing a number of differentiation pathways. In cells where physiologically some of the eight OPA1 isoforms are expressed at adequate levels over the others, probably they provide a specialized support to specific functions (for example exon 4b variants for mtDNA maintenance), thus now working as a “differentiated”, i.e. specialized isoform.

Finally, starting from a condition of OPA1 absence, Del Dotto and colleagues disclose a hierarchy of the mitochondrial hallmarks recovered by OPA1 isoforms. Indeed, mtDNA content, energetics and *cristae* morphology represent the essential “nucleus” of the features that first need to be improved to increase the rate of growth and cellular metabolic efficiency. Subsequent, but not less important for the cellular wellness, there are fusion and mitochondrial network morphology, which “revolve” around the indispensable core of the mitochondrial features (Fig. 3A).



**Fig. 3.** Mitochondrial features hierarchically recovered by OPA1. Figurative representation of the mitochondrial phenotypes recovered by OPA1. (A) The maintenance of the central 'nucleus', comprising the mtDNA content, energetics and *cristae* organization, is mandatory for the cell life, while the mitochondrial dynamics represents a flexible feature which "revolves" around the core, all together generating a hierarchy of distinctive hallmarks necessary for cell wellness. Any of the eight OPA1 isoforms can support the three essential functions, whereas the isoforms generating both l- and s-forms only can sustain the mitochondrial dynamics. (B–C) S-forms are mostly efficient in preserving the energetics whereas l-forms are fusion competent. The pale colors indicate functions carried out with low efficiency by l- or s-forms, respectively.

#### 4. Long and short forms in mitochondrial dynamics and bioenergetics

##### 4.1. Long and short forms in bioenergetics and *cristae* structure

Genetic depletion of Opa1 leads to a drastic reduction of mitochondrial energetic efficiency, in terms of both cell viability and oxygen consumption rate (OCR) or RCS and complex V assembly, strictly dependent on perturbation of mtDNA content and *cristae* organization [33,34]. Conditional ablation of Opa1 alters *cristae* shape and mitochondrial morphology, affecting the amount of assembled RCS, but preserving mtDNA content and translation [3]. Thus, a direct link can be established between *cristae* shape and RCS stability, impacting on respiratory efficiency, further supported by the data obtained in the mouse model of Opa1 mild over-expression, in which increased OPA1 levels promote *cristae* tightening, RCS assembly and mitochondrial energetic function [3]. It remains open and debated the specific role of l- and s-forms alone in recovering the mitochondrial respiratory competence and *cristae* organization. In Opa1-null cell models expressing the l- or s-forms alone, both forms present full and equivalent competence for mitochondrial energetic maintenance (i.e. cell viability in galactose medium, OCR and RCS organization) [34]. Furthermore, both l- or s-forms alone are similarly effective in keeping the *cristae* density and width, and *cristae* junction density [34]. In the same cell models, the s-form alone is more efficient than the l-form in rescuing bioenergetics (i.e. mitochondrial respiration and RCS organization) [33]. L-form indeed exhibits limited OCR and reduced amount of assembled RCS, de-

spite having similar mtDNA amount and *cristae* organization [33] (Fig. 3B–C).

Conversely, by manipulating OMA1 and/or YME1L, a different scenario emerges in vitro. Oma1 null cells display normally shaped *cristae*, whereas Yme1l null cells, where there is a decrease of l-forms and an accumulation of s-forms by Oma1 activation, present a dramatically disturbed *cristae* morphology [35]. Mitochondria lacking both Yme1L and Oma1, characterized by only l-forms, preserve normal *cristae* morphology [35]. Similarly, the knockdown of proteins inducing an unbalance toward OPA1 s-forms, such as HIGD1A, ROMO1, AFG3L2, PHB2 or DNAJC19, leads to disorganized *cristae* structure [36–40]. Thus, models with manipulation of the proteases involved in OPA1 processing support the idea that l-forms are competent for *cristae* maintenance, whereas s-forms are not. However, the observed phenotypes could be attributed directly to the loss of function of these proteins, instead of OPA1 l-/s-forms imbalance. Supporting this view, in vivo l-form stabilization by OMA1 depletion promotes cells survival without restoring *cristae* morphology. For example, Oma1 deletion prevents brain atrophy in *Phb2*-knockout mice [41] and cardiomyopathy in mice with a heart-specific *Yme1l*-knockout [42], without regulating *cristae* morphology.

A model has been proposed where s-forms, lacking the TM domain for IMM anchoring, would require prohibitins, that provide a favorable lipid environment, necessary for membrane binding and *cristae* maintenance [34]. An alternative model suggests that the s-forms might act as a scaffolding passive structure, on which the *cristae* membrane is wrapped and the mtDNA anchored [33]. Such structural frame would possibly encompass components of the mitochondrial contact sites and



*cristae* organizing system (MICOS), recently identified as a master regulator of mitochondrial shape and organization [43,44]. Interestingly, OPA1 was demonstrated to directly interact with the MICOS subunits Mic60/mitofilin and Mic25/CHCHD6 [45]. Further detailed proteomics and genetics studies placed OPA1 as epistatic to Mic60/mitofilin in the biogenesis and remodeling of *cristae* junctions, suggesting the GTPase as the exclusive regulator of *cristae* shape [46]. Finally, in MICOS-deficient cells the phosphatidylethanolamine and cardiolipin metabolism were deranged [47,48], revealing a novel link between MICOS and the mitochondrial membrane properties and phospholipid homeostasis.

Considering the crucial relation between OPA1 and cardiolipin in the membrane fusion process [49], it is plausible that this interaction may contribute to *cristae* shaping. The emerging scenario seems therefore far more complex than predicted, involving a number of protein interactions to stabilize the *cristae* structure, with OPA1 operating as a central hub at the IM in close contact with the MICOS complex, which in turn is intimately linked also to membrane lipid environment, thus influencing the properties of the IM, its curvature and fusion.

#### 4.2. Long and short forms in mitochondrial fusion and morphology

Although the concept that an interconnected mitochondrial network results from an efficient fusion activity is generally accepted, recently it emerged that fusion and network morphology are not always synonymous and need to be analyzed separately (Fig. 4).

Currently, several studies agree on the fact that the l-form alone is fusion competent. Despite it has been proposed a model where the proteolytic processing of OPA1 l-forms promotes OXPHOS-induced fusion [50], in the Oma1/Yme1l double knockout MEFs, where the formation of s-forms is blocked, the presence of l-forms alone is sufficient to fuse mitochondria [35]. Two recent papers demonstrate that the expression of an un-cleavable isoform 1 in Opa1<sup>-/-</sup> MEFs allows for normal mitochondrial fusion [33,34]. Furthermore, by using an in vitro membrane fusion assay, it was clearly shown that OPA1 l-form on one side of the membrane and cardiolipin on the other side, are the minimal components sufficient and necessary for fusion [49].

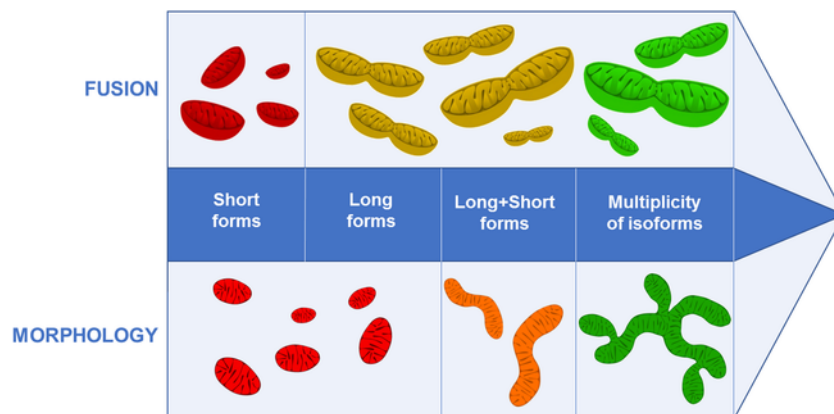
Concerning the involvement of s-forms in fusion, the scenario is less clear. Anand and colleagues suggest that they are involved in mitochondrial fission, given that the expression of a chimeric AIF-s-form fails to modify the fusion rate and that the GTPase-inactive construct co-localizes with sites of mitochondrial division [35]. In this regard, it has to be evidenced that the existence of a trans-membrane domain in the AIF-short form may perturb the function of a soluble short form. Indeed, in vitro experiments demonstrated that although a recombinant OPA1 s-form can tubulate membranes, it is unable to induce membrane

fusion [51]. The recent in vitro membrane fusion assay confirms that s-forms alone are not sufficient to promote fusion, but are involved in this process. In fact, the addition of s-form accelerates the l-form-dependent fusion activity and promotes liposome binding, suggesting that s-form may help fusion by supporting a bridge between l-form and cardiolipin on opposite membranes. However, when s-form is artificially anchored to the membrane by a lipid tail, it shows a partial but significant capacity to stimulate membrane fusion [49]. Similarly, s-form expressed in Opa1<sup>-/-</sup> MEFs presents a minimal, but significant, fusion capacity [33,34]. It has been proposed that the minimal fusogenic activity of s-forms may be a cellular mechanism to reduce fusion, allowing fission to prevail [33]. In fact, it has been observed that after the addition of the uncoupler CCCP, which triggers the cleavage of the l-form, there is a complete fragmentation of the mitochondrial network [22].

While the majority of the studies agree with the fact that s-form is insufficient to maintain a filamentous mitochondrial network [33,34,52], the role of l-form alone remains controversial. It has been observed an interconnected mitochondrial network in Oma1/Yme1l double knockout MEFs or cardiomyocytes, where only the l-forms are present [35,42]. Conversely, the mitochondrial network remains completely fragmented after the expression of an isoform 1 long in Opa1<sup>-/-</sup> MEFs and the l- and s-forms together are able to ameliorate the mitochondrial network morphology [22,33,34]. Indeed, in rat retinal cells the overexpression of isoform 1 long prevents cell death and fragmentation of mitochondrial network induced by ischemia-reperfusion (I/R) injury, re-equilibrating the accumulation of OPA1 s-forms [52]. It is possible that the divergent results of l-forms derive from the different cellular conditions, where the presence of the eight OPA1 isoforms, although all l-forms, may be more efficient in comparison to the single one expressed in MEFs. Indeed, one isoform generating both l- and s-forms induces a partial rescue of the morphology in Opa1<sup>-/-</sup> MEFs, whereas expression of at least two isoforms with a balanced l and s-form ratio is required for a full recovery of the mitochondrial network [33].

#### 5. Therapeutic use of single OPA1 isoforms in pathology

The recent findings and knowledge of the mechanistic function of the OPA1 protein and related isoforms offer a starting point for development of more precise and defined therapeutic prospective. Besides the use of idebenone in DOA patients [53], there is great expectation for the gene therapy approach, aimed at complementing the OPA1 null allele in patients with haploinsufficiency leading to DOA. The existing trials on Leber's congenital amaurosis (trial NCT00999609) and Leber's



**Fig. 4.** OPA1: mitochondrial fusion versus mitochondrial morphology. Cells having s- or l-forms only present a completely fragmented network, although l-form alone is fusion competent. A partial recovery of mitochondrial network morphology is achieved by any isoform generating l- and s-forms together. Only the multiplicity of OPA1 isoforms guarantees the fully rescue of both mitochondrial fusion and morphology.

hereditary optic neuropathy (trials NCT02652767 and NCT02652780) have shown how the eye is an excellent tissue to be targeted by gene therapy. So far, OPA1 isoform 1 has been considered the best to be expressed, being the most abundant in HeLa cells [20] and having shown a protective effect. Indeed, a mild overexpression of isoform 1 protects mice from muscular atrophy, ischemic damage and hepatocyte apoptosis [54] and efficiently ameliorates the phenotype of *Ndufs4*<sup>-/-</sup> and *Cox15*<sup>sm/sm</sup> mice, by improving *cristae* ultrastructure, RCSs organization and respiration [55]. Recent results highlighted that the optimal isoform to be used for gene therapy must satisfy specific requirements, depending on the tissue target [33]. It is now mandatory to study in human RGCs the profile of isoforms expression and which isoforms might be altered by OPA1 pathogenic mutations, in particular how OPA1 levels and l-/s-forms ratio are affected under pathological condition. Considering that all the eight isoforms, if slight overexpressed, show similar ability to recover mtDNA-*cristae*-energetics but not network dynamics and that they generate different l- and s-forms, the optimal choice should consider the isoform that will restore the appropriate OPA1 amount and the balance between l-/s-forms. In this perspective, it will be key to develop a patient-specific strategy to study the OPA1 alterations caused by that particular mutation in that specific individual background, and then choose the isoform with the best potential therapeutic effect.

## Transparency document

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

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