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Review

Mechanistic perspective of mitochondrial fusion: Tubulation vs. fragmentation ☆

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ABSTRACT

Mitochondrial fusion is a fundamental process driven by dynamin related GTPase proteins (DRPs), in contrast to the general SNARE-dependence of most cellular fusion events. The DRPs Mfn1/Mfn2/Fzo1 and OPA1/ Mgm1 are the key effectors for fusion of the mitochondrial outer and inner membranes, respectively. In order to promote fusion, these two DRPs require post-translational modifications and proteolysis. OPA1/ Mgm1 undergoes partial proteolytic processing, which results in a combination between short and long isoforms. In turn, ubiquitylation of mitofusins, after oligomerization and GTP hydrolysis, promotes and positively regulates mitochondrial fusion. In contrast, under conditions of mitochondrial dysfunction, negative regulation by proteolysis on these DRPs results in mitochondrial fragmentation. This occurs by complete processing of OPA1 and via ubiquitylation and degradation of mitofusins. Mitochondrial fragmentation contributes to the elimination of damaged mitochondria by mitophagy, and may play a protective role against Parkinson's disease. Moreover, a link of Mfn2 to Alzheimer's disease is emerging and mutations in Mfn2 or OPA1 cause Charcot–Marie–Tooth type 2A neuropathy or autosomal-dominant optic atrophy. Here, we summarize our current understanding on the molecular mechanisms promoting or inhibiting fusion of mitochondrial membranes, which is essential for cellular survival and disease control. This article is part of a Special Issue entitled: Mitochondrial dynamics and physiology.

1. Introduction

Mitochondria are very dynamic organelles, whose morphological changes are achieved by constantly occurring fusion and fission events [1]. Loss of mitochondrial fusion is characterized by the presence of fragmented mitochondria, produced by ongoing fission, which contrasts to the characteristic network of most cellular types. Mitochondrial fission not only allows proper distribution of mitochondria, for example to cope with local ATP demands, but also contributes to selective removal of damaged organelles. Fusion of mitochondrial membranes, in turn, allows organelle content mixing and prevents mitochondrial DNA loss, facilitating maximal ATP production. Mitochondrial fusion is particularly important in the nervous system, helping neurons to meet the high energy demands for proper neuronal function, by diluting out injury and dysfunction to which each individual mitochondrion is subject [2,3]. Mitochondrial fusion therefore plays a protective role, preventing these deficiencies from damaging the entire neuron while maintaining an adequate level of bioenergetic capacity [4,5].

Many studies have implicated impairment of mitochondrial function as a contributor to both common and rare neurodegenerative diseases. Early discoveries showed the direct role of the central fusion components in the autosomal-dominant optic atrophy (ADOA) and in the Charcot–Marie–Tooth type 2A neuropathy (CMT2A) [6–9]. More recently, a link between mitochondrial fusion and the most common neurodegenerative diseases of the aging population, and also a link with cardiopathies, were observed [10–12]. Interestingly, the ubiquitylation of mitofusins by the Parkin E3 ligase (often mutated in Parkinson's disease patients) appears to contribute in targeting damaged mitochondria for degradation, which could protect against Parkinson's disease [13–15]. Because these topics are detailed in accompanying reviews in this issue, here we focus on the molecular mechanisms that either prevent or promote mitochondrial fusion. This leads to the opposite outcomes of mitochondrial tubulation or fragmentation. In this respect, it is interesting to notice that mitochondrial fusion recently joined a group of fundamental processes, such as transcription or cellular trafficking, which are controlled by ubiquitylation.

Mitochondrial fusion occurs if two tips or one tip and one tubule come together. The field emerged essentially with pioneer observations of fusion events and with the identification of the first protein required for fusion, Fzo, in fly [16–18]. Studies initially based on genetic screens, coupled with observations of mitochondrial morphology, allowed the identification of most proteins involved in mitochondrial

fusion [19,20]. Subsequently, topological studies and analysis of the physical interactions between the different proteins have allowed the field to progress. In addition, the development of cell free in vitro tethering and fusion assays was of outmost importance [21–25]. Interestingly, the discovery of the yeast and mammalian homologs of Fzo [18,26–28] was an early indicator for common mechanisms and indeed the basic principles in mitochondrial fusion have proven to be conserved.

2. The key mediators of mitochondrial fusion

While the vast majority of the membrane fusion events in a cell are performed by SNAREs, fusion of mitochondria and of the endoplasmic reticulum (ER) depends on dynamin-related proteins (DRPs) [29–32]. DRPs are a special class of GTPases, which provide the mechanical forces necessary for membrane remodeling [33,34]. They are best known for their role in membrane scission events, particularly during endocytosis and certain other membrane budding events, and also perform fission in mitochondria and peroxisomes [35,36]. The key DRPs involved in mitochondrial fusion are conserved in yeast, worms, flies, mice and humans (Table 1).

2.1. Mitofusins, the DRPs in the OM

Mitofusins, the DRPs that mediate fusion of mitochondrial outer membranes (OM), are termed Mfn1 and Mfn2 in mammals, Fzo and Marf/Dmfn in flies, FZO-1 in the worm and Fzo1 in budding yeast. In contrast to the founding member identified, FZO, shortly expressed during fly spermatid development [18], all the other mitofusins are constitutively expressed ubiquitous mitochondrial proteins, in both males and females [26,28,37–40]. Loss of Fzo1 in yeast leads to loss of mitochondrial DNA and consequently loss of mitochondrial translation, resulting in respiratory incompetence [26,27]. In flies Fzo loss leads to male sterility [18], and in mice Mfn1 KO or Mfn2 KO is embryonic lethal due to a placental defect [4]. Moreover, if Mfn2 is depleted only after placental formation, mice show impaired cerebella development and lethality at day 1 post-birth [5]. In contrast, mice are normal if depleted of Mfn1 only after placental formation [5], suggesting that after this stage Mfn1, but not Mfn2, is dispensable. Isolated cells lacking both Mfn1 and 2 showed severe cellular defects, including poor cell growth, widespread heterogeneity of mitochondrial membrane potential and decreased cellular respiration, whereas single Mfn KO escaped major cellular dysfunction [41]. In addition, Mfn2 depletion leads to a loss or reduction of membrane potential [4,42].

Mitofusins are localized throughout the mitochondrial network [26,28,37,39,40]. They are anchored to the OM by two transmembrane domains, and their N-terminus and C-terminus are exposed to the cytoplasm [18,26,27,37,43] (see Table 1 and Fig. 1). The N-terminus comprises the GTPase domain, followed by one coiled-coil heptad repeat (HR1). Moreover, yeast, worm and fly mitofusins have one additional coiled-coil motif upstream of the GTPase domain (HRN) (see Table 1). Although no equivalent coiled-coil is present in mammalian mitofusins, the N-terminal region upstream of the GTPase domain in Mfn2 is also essential for its function [44]. In turn, the C-terminal domain of mitofusins possesses one additional coiled-coil heptad repeat (HR2). All HR domains are required for mitofusin function, at least in yeast [45]. Importantly, mutations in the conserved GTPase P-loop or switch motifs abolished mitochondrial fusion in all organisms, indicating that both GTP binding and hydrolysis are conserved requirements [4,18,26,45–47].

As expected for DRPs, mitofusins self-assemble [27,39] and in mammals as in yeast, GTP induced changes in the three oligomerization states detected [47,48]. P-loop mutants were exclusively present in a lower molecular weight form, being the wild-type Fzo1 or Mfn1 recovered in a complex with a middle molecular weight [47,48].

After *trans* associations, i.e. associations between two mitochondria, the middle molecular weight complex then shifted to the higher oligomerization state [47,48]. Recent studies in yeast clearly demonstrated that the smaller complex corresponds to the monomeric form of the protein, whereas the middle complex represents a dimer in *cis* (in the same mitochondria), formed upon GTP binding [47]. The oligomeric state of the bigger *trans* complex has not been clearly defined, but was compatible with the formation of a tetramer in *trans* [47].

In addition to the oligomerization of full-length mitofusins, their N-terminal HR1 and C-terminal HR2 interact with each other [37,45]. This interaction is GTPase dependent, because it is not formed in GTP binding or hydrolysis mutants [44,45], and also depends on an intact N-terminal domain [44]. Moreover, co-expression of non-overlapping halves of Fzo1 domains partially complements the wild-type protein, showing that different domains can be provided in separate molecules [45].

2.2. OPA1/Mgm1, the DRP promoting fusion in the IM

The key mediator of inner mitochondrial membrane fusion is called Mgm1 in budding yeast, Msp1 in fission yeast, eat-3 in the worm and OPA1 in flies and mammals [6,7,49–59]. OPA1 is expressed in all tissues analyzed and both in vivo and in isolated mitochondria Mgm1/OPA1 is localized to discrete foci in interface regions of the IM [22,53,60,61]. Loss of Mgm1 leads to respiratory incompetence in yeast due to mitochondrial DNA loss and OPA1 KO in mice is embryonic lethal [49,62,63]. In addition, OPA1 repression in mammals decreases cell growth and oxygen consumption [41,64–67]. Mutations in OPA1 cause ADOA, characterized by progressive bilateral blindness due to the loss of retinal ganglion cells and optic nerve deterioration [6,7,68]. Consistently, heterozygous mutant OPA1 mice show a visual impairment resembling the human ADOA [62,63]. OPA1 mutations are associated with multiple deletions in mitochondrial DNA and also with other neurological conditions adding to ADOA, called "OPA1 plus" phenotype. This consists of chronic progressive ophthalmoplegia, ataxia, sensironeuronal deafness, sensory-motor neuropathy and myopathy [68]. Moreover, in addition to its fundamental role in mitochondrial fusion, OPA1/Mgm1 is also required for cristae formation [22,65,69–72]. Loss of OPA1/Msp1 leads to cell death [64,65,73] and increases sensitivity to apoptosis, which was proposed to occur via increased release of cytochrome *c* due to widened cristae junctions [65,66,67,70,72,74–76]. The structural role of OPA1/Mgm1 in cristae formation occurs via oligomeric self-interactions and was suggested to be independent of ongoing mitochondrial fusion, because it was not abolished in the absence of Mfn1 [75]. However, inactivation of the main fission machinery component, Dnm1, reverses the cristae morphology phenotype of yeast Mgm1 mutants [69].

OPA1/Mgm1 is present in the IM with long, membrane anchored, and short soluble forms, both required for IM fusion [77,78]. These short and long isoforms constitute a complex pattern regulated both post-transcriptionally and post-translationally. In mammals, alternative splicing of the mRNA creates several long isoforms, which are processed to yield several short isoforms [67,79,80], whereas in yeast there is only one long and one short isoform [77,81,82]. All OPA1 variants possess a mitochondrial targeting sequence, cleaved upon import. This sequence is followed by a transmembrane segment that anchors the long isoforms in the inner membrane (IM), with the bulk of the protein facing the intermembrane space (IMS) (Table 1 and Fig. 1). The short isoforms are constitutively generated by further proteolytic maturation at the S2 cleavage site (Table 1) [83]. Only a fraction of the long isoforms is cleaved, thereby producing equimolar amounts of long and short isoforms. This cleavage is performed by Pcp1 in yeast [77,81,82] and was found to depend on cellular ATP levels [84]. In mammals, it was shown that constitutive processing at the S2 cleavage site depends on YME1L [78,85]. However, other

Table 1

Domain structure of mitochondrial fusion proteins. Representation of the function, domain structure and localization of the proteins involved in mitochondrial fusion, i.e. dynamin-related proteins (DRPs) and accessory proteins (accessory). In case of mammalian proteins, the *Homo sapiens* homolog is represented. Dm, *Drosophila melanogaster*; Ce, *Caenorhabditis elegans*; Sc, *Saccharomyces cerevisiae*. The triangle on OPA1/Mgm1 indicates cleavage sites.

Function	Protein	Structure	Organism	Localization	Reference
OM fusion	Mfn1	GTPase HR1 HR2 769	mammals	OM	4, 5, 28, 41
	Mfn2	GTPase HR1 HR2 757	mammals		
	Marf	GTPase HR1 HR2 810	Dm		18, 38
	Fzo	GTPase HR1 HR2 718	Dm		
	Fzo-1	HRN GTPase HR1 HR2 775	Ce		55-57, 142
	Fzo1	HRN GTPase HR1 HR2 855	Sc		21, 26, 27
	MIB	394	mammals	Cytoplasm	150
	Bax	B B B 192	mammals		25, 140
	Mdm30	F-Box 598	Sc		47, 110-112, 124
	MitoPLD	252	mammals	OM	126, 131, 137
	Zucchini	253	Dm		131
OM + IM fusion	Ugo1	501	Sc	OM	47, 102-104, 109
IM fusion	OPA1	MTS HR GTPase Middle GED 950	mammals	IM	54, 62, 63
	Opa1	MTS HR GTPase Middle GED 972	Dm		58, 59
	Eat-3	MTS HR GTPase Middle GED 964	Ce		55, 56, 142
	Mgm1	MTS HR GTPase Middle GED 881	Sc		22, 53, 89, 91, 92
	YME1L	MTS ATPase Protease 773	mammals		78, 85
	Rhomboid-7	MTS 346	Dm		59, 98
	Pcp1	MTS 351	Sc		77, 81, 82

GTPase GTPase domain | GED GTPase effector domain | Protease proteolytic domain
HR heptad repeat domain | Middle middle domain | B BH domain
transmembrane domain | ATPase ATPase domain
F-Box heptad repeat domain | MTS mitochondrial targeting sequence

proteases are likely involved, like OMA1, which also affects the constitutive levels of some OPA1 isoforms [86–88]. Downstream of the S2 cleavage site, all OPA1/Mgm1 forms possess one coiled-coil domain, followed by the typical DRP motifs: GTPase, middle and GED domains (Table 1), all essential to promote IM fusion [22,69,89,90]. This combination between short and long isoforms certainly contributes to IM fusion regulation, because Mgm1 cleavage into the short isoforms results in activation of its GTPase activity, which is only required on the short isoforms [91,92]. In turn, long isoforms could be required to target the short forms via self-interactions [22,91,92].
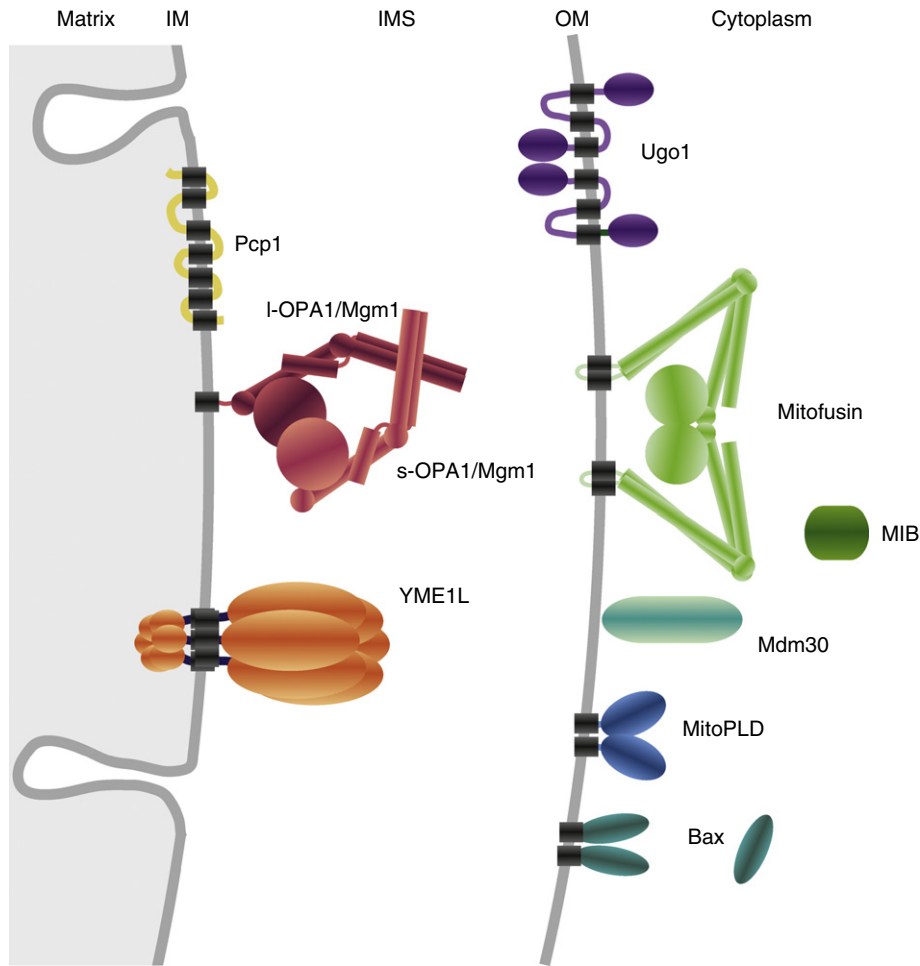
3. Mitochondrial fusion accessory proteins

In addition to the DRPs, mitochondrial fusion depends on several other proteins (Fig. 1 and Table 1). Fusion of the IM requires Pcp1/Rho-7 in yeast and flies, YME1L in mammals and Ugo1 in yeast. In turn, OM fusion is modulated by Ugo1 and Mdm30 in yeast,

MitoPLD/Zuc in mammals and flies, and Bax and MIB in mammals. Despite the high conservation between mitofusins, they are not interchangeable between yeast, fly and mammals [39], possibly due to the lack of conservation of some of the accessory proteins discovered so far.

3.1. Pcp1/Rho-7/PARL, IM fusion activator

Pcp1 in yeast, Rho-7 in fly and PARL (presenilin-associated rhomboid-like) in mammals are the mitochondrial rhomboid proteases. They possess seven membrane-spanning regions and are localized to the IM [93,94]. Rhomboid proteases comprise a conserved protein family of integral membrane serine endoproteases, which generally clip substrate proteins within their membrane-spanning segment [95–97]. Pcp1 modulates mitochondrial morphology in yeast because it processes Mgm1 [77,81,82]. Although PARL expressed in yeast can process Mgm1 [82], its role in OPA1 processing

Fig. 1. Topology of mitochondrial fusion proteins. The topology and localization of the proteins involved in mitochondrial fusion, shown in Table 1, are depicted. The proteins are represented in their predominant oligomeric state.

is still controversial. Indeed, it was reported that PARL is involved in the release of short OPA1 isoforms from mitochondria during apoptosis [76], but OPA1 processing occurs in the absence of PARL/Rho-7 [85,98].

3.2. YME1L, more than a quality control regulator

The *i*-AAA protease is one of the ATP-dependent quality control AAA proteases responsible for the recognition and degradation of miss-folded proteins in mitochondria [99–101]. Located in the IM, it has its catalytic center facing the inter membrane space (Fig. 1). It is a homohexamer composed of Yme1/YME1L subunits, which form a proteolytic chamber allowing proteolysis to occur in a sequestered environment. In mammals, YME1L is important for IM fusion because it is responsible for cleavage of the long OPA1 isoforms at the S2 site [78,85].

3.3. Ugo1, the coordinator of OM and IM fusion

Ugo1, which means "fusion" in Japanese, is an OM protein that is essential for fusion of yeast mitochondria both in vivo [102] and in vitro [103]. It is a modified carrier protein [104], containing three TM domains, which dimerizes [103]. This creates a complex with 6 TM domains, typical of carrier proteins. However, a transporter function has not yet been assigned to Ugo1. Recent studies revealed that the Ugo1 protein interacts with the recently identified MICOS/MITOS/MINOS complex, a large contact site scaffold-like protein complex, which controls mitochondrial architecture and biogenesis

[105–107]. Importantly, Δ*ugo1* cells had a drastic reduced number of cristae and cristae junctions [107].

Interestingly, pioneer microscopy studies reported that mitochondrial fusion initiates at sites of close apposition of the IM and OM [108]. Consistently, biochemical fractionation revealed that Fzo1 localizes at contact sites of the IM with the OM [26]. This localization depends on the small 6–10 aa loop of Fzo1 present in the IMS [43] and is required for mitochondrial fusion [43]. Moreover, OPA1 requires Mfn1 to stimulate mitochondrial fusion, supporting a conserved interdependence of OM and IM fusion.

The Ugo1 protein was proposed to coordinate yeast OM and IM fusion, which occur simultaneously in vivo [31,89], due to its physical interactions with both Fzo1 and Mgm1 [89,109]. Ugo1 interacts directly with Fzo1, independently of Mgm1, via its cytosolic N-terminal domain. Furthermore, it also interacts with Mgm1, independently of Fzo1, via its C-terminal and IMS domain [89,109]. In vitro fusion assays revealed that indeed Ugo1 is required for fusion of both mitochondrial membranes [103]. Ugo1 might function as an adaptor between IM and OM DRPs [103], because the protein complex between Fzo1 and Mgm1 depends on Ugo1 [109]. However, physical interactions of Ugo1 with each DRP are not sufficient to drive mitochondrial fusion [103], as shown with *UGO1* mutant strains incapable to fuse. This suggests that the role of Ugo1 goes beyond that of a simple adaptor. Although not conserved, a structural or functional ortholog of Ugo1 might exist among the many members of the transport/carrier protein family in humans. In fact, mammalian OM and IM DRPs also interact physically.

In contrast to the DRPs, Ugo1 fusion incompetent mutants were largely capable to fuse with wild-type mitochondria [103]. This indicates that a functional Ugo1 is only required on one mitochondrial partner. Moreover, Ugo1 was distinctly required for either IM or OM fusion: mutations in the IMS domains of Ugo1 blocked mitochondrial fusion although OM fusion was not impaired. Conversely, additional mutations in the cytoplasmic region of Ugo1 impaired OM fusion. Importantly, these *ugo1^{ts}* mutants have been useful for proposing a role for Ugo1 following tethering of either mitochondrial membrane [103]. Thus, Ugo1 could be required for mixing of the lipid membranes, the last step required for membrane fusion. On the other hand, complete absence of the Ugo1 protein impaired Fzo1 dimerization in *cis*, thus affecting mitochondrial fusion before the tethering step [47]. Therefore, Ugo1 appears to act at both early and late stages of mitochondrial OM fusion.

3.4. Mdm30, the post-translational modifier of Fzo1

Mdm30 is a cytosolic, mitochondrial-associated F-box protein that binds and ubiquitylates Fzo1 [110–112]. The covalent binding of ubiquitin to its substrate is catalyzed by three sequential steps, starting with ubiquitin activation by E1 enzymes, its conjugation to the second set of enzymes called E2, being finally transferred to its target by the substrate-specific E3 ligases [113]. F-box proteins constitute a special class of E3 ligases, called SCF complexes (Skp1, Cdc53 and F-box), in which the F-box subunit is responsible for substrate specificity [114]. In addition to Fzo1, two other substrates of the SCF^{Mdm30} have been described so far, Mdm34 and Gal4. Mdm34 is one of the OM components of the ERMES complex, which promotes physical associations between the ER and mitochondria [115–117]. Gal4 is a nuclear transcription factor responsible for the activation of the galactose metabolism pathway [118,119]. Moreover, Mdm30 has been implicated in the stimulation of the nuclear export of certain mRNAs [120].

Mdm30 acts at a late stage of mitochondrial OM fusion via ubiquitylation and proteolysis of Fzo1, after *trans* associations and GTP hydrolysis of Fzo1 [47,111,112,121–124]. Therefore, Mdm30 does not operate as a regulator of Fzo1 quality control but rather acts on Fzo1 at the last step in the OM fusion process. Absence of Mdm30 strongly impairs mitochondria fusion [110,125] and leads to the clustering of mitochondrial fragments, as revealed by electron microscopy images [121].

3.5. MitoPLD/Zuc, the role of the lipids for OM fusion

MitoPLD belongs to the phospholipase D superfamily, being most closely related to prokaryotic PLDs called Nuc. In contrast to the classical mammalian PLD family members, MitoPLD possesses an HKD-half catalytic site, which by dimerization creates a functional enzyme [126]. It cleaves the mitochondrial-specific lipid cardiolipin to generate PA, a signaling lipid which activates SNARE-driven fusion events [127]. MitoPLD is anchored to the mitochondrial OM, facing the cytoplasm, and was first reported to promote mitochondrial OM fusion [126]. MitoPLD downregulation or use of enzymatic inactive variants results in mitochondrial fragmentation by inhibition of mitochondrial fusion. In contrast, MitoPLD overexpression leads to mitochondrial aggregation, dependent on the presence of mitofusins. Similar to Mfn1 overexpression, these aggregates consist of closely associated mitochondrial fragments. However, in each case, the spacing between the fragments is different. Overexpression of Mfn1 leads to fragments separated by 16 nm, whereas in the aggregates driven by MitoPLD overexpression mitochondrial spacing is reduced to 6 nm [126,128]. This suggests a role for MitoPLD acting in membrane merging following tethering by mitofusins, perhaps by hydrolysis of cardiolipin in the opposing membrane [126]. Interestingly, cardiolipin biosynthesis was downregulated immediately post mitochondrial

fusion, rising again at later time points, suggesting a possible compensatory feed-back mechanism [129]. However, whether MitoPLD requires GTP hydrolysis by mitofusins, and which mechanism is underlying the precise role of MitoPLD in mitochondrial fusion, is currently unknown [130]. Interestingly, PA generation by MitoPLD recruits Lipin 1b, which converts PA to DAG, which in turn promotes mitochondrial fission [131]. This supports previously reported spatio-temporal connections between mitochondrial fusion and fission [132,133]. In addition to its role in mitochondrial fusion, MitoPLD is also called zucchini (Zuc) in drosophila and is required for piRNA generation, critical for germ-line development [134,135]. Zuc/MitoPLD absence leads to a failure to suppress transposon mobilization, inducing DNA-damage and check-point induced meiotic arrest, leading to apoptosis and sterility [131,136,137].

3.6. Bax, stimulation versus inhibition of mitochondrial fusion

Bax is a pro-apoptotic protein from the Bcl-2 family that regulates mitochondrial fusion both positively and negatively. Upon apoptotic stimuli, cytosolic Bax migrates to mitochondria, inserting itself and oligomerizing in the OM thereby punching holes in it, allowing cytochrome c to leak out and inducing apoptosis [138]. In general, mitochondrial fusion protects against apoptosis, perhaps via maintenance of cristae integrity by OPA1, avoiding cytochrome c release [75]. Consistently, activation of apoptosis inhibits mitochondrial fusion [139]. In contrast, in healthy cells, a stimulatory role of mitochondrial fusion from pro-apoptotic proteins like Bax and tBID has also been reported [25,140–144]. Bax interacts with Mfn2 and in Bax/Bak double knockout cells the focal distribution and oligomerization of Mfn2 are impaired [140]. Consistently, Mfn2 overexpression reversed the shorter mitochondrial tubules caused by the lack of Bax and Bak [140,143]. In healthy cells, Bax is constantly cycling between the cytosol and the mitochondria [144]. If this cycling is blocked, Bax loses its ability to stimulate fusion in vitro [25]. In addition, expression of a chimeric protein of Bcl-xL with helix 5 of Bax, which shows stronger binding to Mfn2 compared to Bax, reduces mitofusin mobility in the OM and inhibits mitochondrial fusion [143]. Therefore, transient interactions between Bax and Mfn2 could be required for efficient fusion by modulating Mfn2 assembly or concentration at the fusion sites. Bax could also play a role in membrane sculpting, due to its high propensity to insert in highly curved membranes.

Apart from Bax, several apoptosis related proteins, Bak, Bcl-x and Bcl-2, have been described as physical interactors of mitofusins [145–149], suggesting a general role of Bcl-2 family proteins in the regulation of mitochondrial morphology.

3.7. MIB, a negative regulator of mitochondrial fusion

The MIB protein is important to the maintenance of tubular mitochondria, because its overexpression induces fragmentation and its downregulation promotes extensive tubulation [150]. MIB appears to be a negative regulator of mitochondrial fusion, because it physically interacts with Mfn1 and Mfn2. Consistently, MIB partially fractionates with the membrane-enriched cellular extracts containing mitochondria and ER, although it mostly localizes to the cytoplasm. Intriguingly, if overexpressed, MIB disperses in the cytoplasm while it induces mitochondrial fragmentation. The phenotypes of MIB depend on a GGVG-conserved motif of its coenzyme-binding domain, and are apparently independent of mitochondrial fission. It remains to be analyzed how MIB negatively regulates mitofusin activity. In analogy to its homolog, the VAT-1 protein, regulation could involve the ATPase activity or Ca^{2+}-dependent oligomeric-complex forming activity.

4. Tethering between mitochondrial membranes—tools and evidence

Early observations with overexpression studies suggested that interactions in *trans* between mitofusins lead to apposed mitochondria. Indeed, Mfn1, Mfn2 or Fzo1 overexpression leads to the formation of spherical non-fused but closely associated mitochondrial clusters, which display perinuclear localization in mammalian cells [28,37, 39,111]. Clustering was dependent on the C-terminal coiled coil, but was not abolished after deletion of the GTPase motif [28,37,45]. In addition, mutant forms of Mfn2 [40] or truncated forms of Mfn1 [128] lead to association of fragmented mitochondria. Consistently, mitochondrial fusion both in vivo and in vitro requires mitofusins on adjacent mitochondria [21,128]. Moreover, it appears that only one mitofusin is required on each mitochondrion, because single KO cells could fuse with wild-type cells, whereas no mitochondrial fusion activity could be detected in vivo by mixing double Mfn1 Mfn2 KO with wild-type cells [128].

Compelling evidence for *trans* associations of mitochondria was provided by an in vitro docking assay, based on measurements of the association between isolated mammalian mitochondria labeled with different fluorescent markers [48]. This assay revealed that external expression in wild-type cells of Mfn1, but not of Mfn2, promoted mitochondrial tethering in a GTP-, temperature- and time-dependent manner [48]. A similar assay in yeast showed that Fzo1 also promotes mitochondrial tethering, impaired in GTP binding mutants [124]. Importantly, co-immunoprecipitation studies directly demonstrated that mammalian and yeast mitofusins not only promote *trans* associations of mitochondria but also physically associate in *trans* [47,48]. Differentially tagged mitofusins present on separate populations of OM vesicles or on isolated mitochondria revealed the presence of protein complexes in *trans*. An assay based on electron microscopy observations allowed independent measurement of IM [22] and OM tethering [103]. Mitochondrial tethering is identified in regions of mitochondrial OM or IM association by the presence of membranes tightly aligned, reciprocally deformed and evenly spaced apart [21,103].

5. Tools to measure mitochondrial fusion

Although to date there is no direct demonstration for fusion after reconstitution on liposomes of mitofusins and Mgm1/OPA1, there is good in vivo and in vitro evidence that those are the proteins that promote fusion directly. Several in vivo assays with cellular cultures, and also in vitro assays with isolated mitochondria, are available. They showed complete blockage of mitochondrial fusion in the absence of OPA1/Mgm1, Fzo1 and both Mfn1 and Mfn2 [21,22,25, 26,41,53,66,78,89,90,128,151] and strong impairment in the absence of either Mfn1 or Mfn2 [4,25,40,41].

5.1. In vivo fusion assays

The first assay reported for assessing mitochondrial fusion in vivo was with yeast cells. It is based on mixing of mitochondria labeled with different fluorophores, during mating between two haploid cells, and it is monitored by fluorescence co-localization [17,152]. For mammals, a similar assay is based on PEG-induced or virus-induced whole cell fusion between two differently labeled populations of mitochondria [46,153]. A second assay described for mammalian cells uses a photoactivable GFP, where small populations of mitochondria are fluorescently activated and their subsequent fusion events scored using fluorescent quantification [154]. In vivo fusion assays have demonstrated that fusion is completely inhibited after depletion of $\Delta\psi$ with CCCP, but can occur in rho^0 cells, after ATP depletion, after depolarization of the cytoskeleton and is independent of protein synthesis [46,153].

5.2. In vitro fusion assays

Importantly, the later development of cell free assays for measuring fusion events was of fundamental importance and allowed the definition of discrete steps in mitochondrial fusion. First, an assay using isolated yeast mitochondria labeled with various fluorescent markers allowed the assessment of content-mixing by the co-localization of the fluorophores expressed in different mitochondrial compartments [21]. Moreover, a readily automated and quantitative fusion assay, based on luciferase activity for assessing matrix content mixing of isolated mammalian mitochondria, was recently developed [24]. The critical and initial requirement for mitochondrial fusion is that mitochondria are brought into close contact, presumably to permit tethering and docking. In vitro, this is achieved by a centrifugation step followed by an incubation step on ice. Afterwards, resuspension in the energy regeneration fusion buffer, accompanied by temperature increase and further incubation, allows mitochondrial fusion [23].

In vitro fusion assays revealed that the core requirements for mitochondrial fusion are similar in yeast and mammalian cells [21,24,25]. First, apyrase and non-hydrolysable GTP analogs completely inhibit mitochondrial fusion, directly demonstrating that GTP hydrolysis is essential [21,24,25]. An intact membrane potential is essential, because in the presence of CCCP no fusion was detected. As detailed below, this can be explained by CCCP-induced proteolytic processing and inactivation of long OPA1 isoforms by the OMA1 protease [86,87]. Moreover, valinomycin, and to a lower extent nigericin, also inhibited mitochondrial fusion, indicating that the electric more than the proton gradient is required [21,24]. Both in yeast and in mammals, addition of cytosol to the fusion buffer was not required for mitochondrial fusion, confirming that the core machinery is physically associated with mitochondria. However, in mammals, under certain conditions cytosol addition stimulated or inhibited mitochondrial fusion, suggesting the influence of signaling pathways coming from the cytoplasm [24,25], possibly involving phosphoproteins like PKA [24]. Interestingly, induction of apoptosis inhibited mitochondrial fusion in vitro [24], consolidating previous in vivo data [154,155]. Conversely, in healthy cells, the positive role of Bax in mitochondrial fusion [140] was also recapitulated in vitro, where addition of recombinant bax stimulates Mfn2 mediated fusion [25]. This effect was also observed using an oligomerization defective mutant of Bax, suggesting that the fusion promoting role of Bax is independent of its function in apoptosis.

The first in vitro assay of mitochondrial fusion described, although semi-quantitative, also allows one to separately follow OM and IM fusion events. Both OM and IM fusion required GTP hydrolysis and depended on the presence of the respective DRPs in both mitochondria to be fused [21,22,25]. Moreover, although impairment of Mgm1/OPA1 completely blocked IM fusion, the competence for OM fusion was not affected [22,25]. Interestingly, whereas IM fusion required the addition of external GTP and of an energy regeneration system, low GTP levels were sufficient to promote OM fusion. In addition, OM fusion only needed the proton gradient component (ΔpH) of the IM electrochemical potential, perhaps to maintain membrane integrity, but not the electrical component, in turn required for IM fusion. To clarify the requirements for fusion observed with isolated mitochondria, it would be interesting to separately reconstitute fusion events with purified OM or IM fractions.

6. Molecular mechanisms of mitochondrial fusion

As stated above, fusion of each of the two mitochondrial membranes is not only performed by different machineries but also has different energetic requirements, clearly indicating that the OM and IM DRPs function differently [21]. The need for low GTP amounts in OM fusion in vitro suggests that mitofusins use a different mechanism from the classical DRPs. In contrast, IM fusion could be more similar to

the classical DRP mechanism, because of its requirements for high GTP levels in the in vitro *fusion* assay. Nevertheless, although in classical DRPs the driving force is provided by GTP hydrolysis, the rate observed for GTP hydrolysis by Mgm1 was relatively low [92,156]. Importantly, hints on how these proteins might function are provided by the recently solved crystal structures of bacterial dynamin-like protein (BDLP) and dynamin 1, close homologs to mitofusins and Mgm1/OPA1, respectively [157–160]. The numerous genetic and biochemical findings described above, together with the crystal

structures of homologous DRPs, allow one to extrapolate and propose possible mechanistic models of IM and OM fusion [32,161]. However, it should be noted that how the different nucleotide states and *trans* associations of the DRPs influence the polymer structure, and how this is coupled to membrane fusion, are not yet clear.
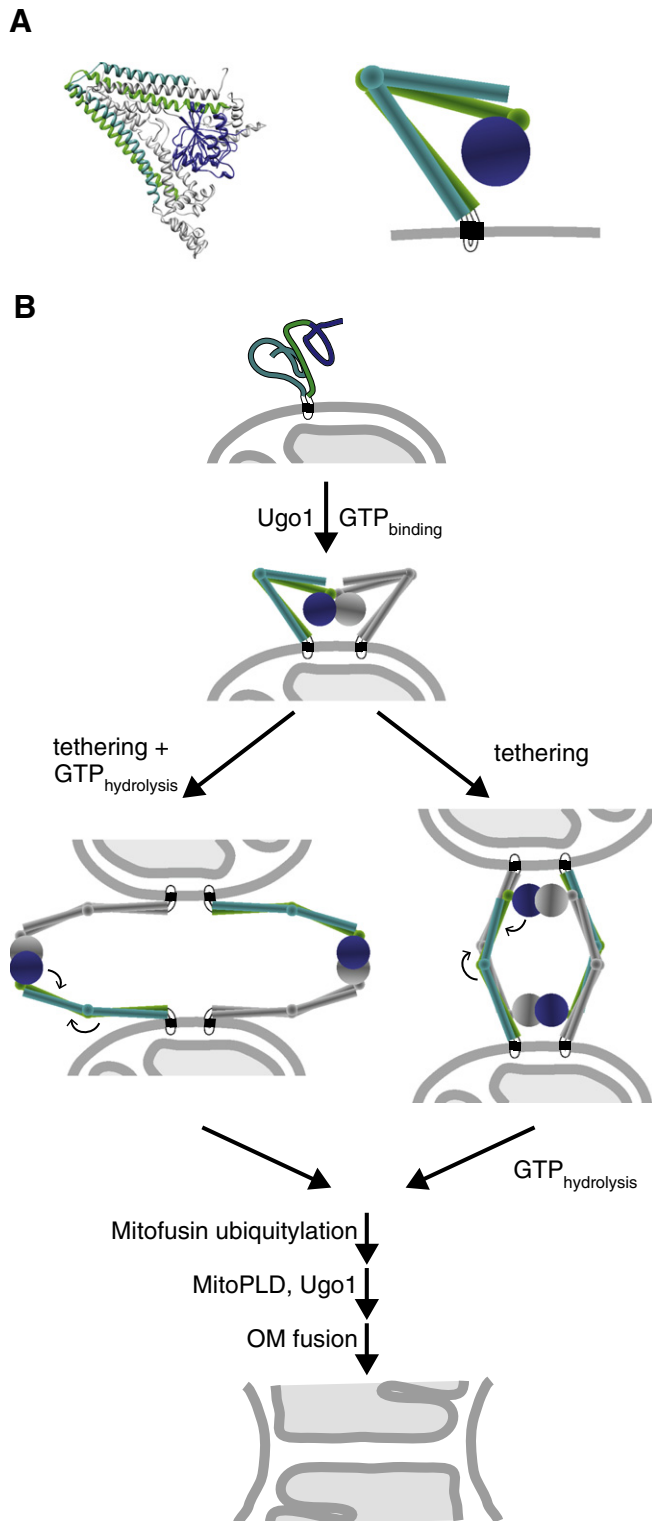
6.1. OM fusion model

Early studies showed that Fzo1 and Mfn1/Mfn2 are involved in mitochondrial docking. Physical interactions between mitofusins in *trans*, allowing the alignment of mitochondrial membranes for fusion events, have been demonstrated on both yeast and mammals [47,48]. In addition, recent work has elucidated the role of different oligomerization states during mitochondrial fusion [47]. Importantly, structural information from BDLP, the mitofusin homolog from cyanobacteria, can be used to further understand mitofusin oligomers [157,158]. In fact, the GTPase domain of mitofusins is most similar to a family of eubacterial GTPases, suggesting that it is derived from an ancestral prokaryote. Although the role of GTP binding and hydrolysis had remained elusive, it is now clearer how the GTPase cycle is coupled to individual fusion states [47]. However, direct tests of the GTPase nucleotide bound state and activity of mitofusins in the different oligomerization states are still missing. After GTP hydrolysis, OM fusion requires mitofusin ubiquitylation in yeast [47,124], which then with the help of Ugo1, drives completion of the fusion process [103]. The current vision of how OMs fuse, where Mfn1 structural arrangements were modeled on BDLP, is summarized in Fig. 2.

6.1.1. Oligomerization of mitofusins—dimerization in cis and further association in trans

As stated, several observations support both *cis* and *trans* associations between mitofusins, consistent with the intragenic interactions observed for the classical DRPs. The crystal structure of BDLP reveals a compact molecule in which the predicted GTPase and coiled-coil domains do not form discrete entities [157]. Moreover, interactions between the GTPase domains promote BDLP dimerization [157,158], consistent with the dimer form observed for *cis* complexes of yeast mitofusin [47]. Not unexpectedly, the structure of BDLP revealed massive changes upon lipid binding, also supported by increase in the oligomeric state of Fzo1 or Mfn1 after *trans* associations [47,48].

How mitofusin dimers, tetramers or possibly higher order structures assemble in *trans* is not yet clear. Initially, structural analysis of the C-terminal coiled-coil of Mfn1 has revealed that it can fold into a dimeric antiparallel helical structure that is 95A long, which was proposed to drive mitochondrial tethering [128]. The integrity of this coiled-coil is important, because if mutated in the full length protein it impairs tubular morphology and mitochondrial fusion

Fig. 2. OM fusion model, integrating the oligomerization and GTPase properties of mitofusins at distinct steps in the fusion process. (A) Structural and schematic model of human Mfn1. Human Mfn1 was modeled on the bacterial homolog BDLP using i-tasser (C-score −0.80) [157,199]. The N-terminal heptad repeat domain HR1 is depicted in green, the C-terminal HR2 is depicted in turquoise, the GTPase domain in blue and the transmembrane domains in black. (B) Model of mitochondrial OM fusion. Mitofusins are integrated into the OM by two transmembrane domains exposing N- and C-terminus to the cytosol. Upon GTP binding mitofusins dimerize in one membrane, i.e. in *cis*, which in yeast depends on Ugo1. Mitochondrial tethering of opposing organelles occurs by mitofusin *trans* interactions, presumably GTP hydrolysis-independent. Two alternative mitofusin *trans* interactions are shown, both speculative. In the model on the left side, mitofusins interact in *trans* via their GTPase domains, in accordance with the interactions observed between the GTPase domains in the open conformation of BDLP [158]. In an alternative model, depicted on the right side, mitofusin dimers interact in *trans* involving anti-parallel associations between the C-terminal helices, as previously proposed [128]. Subsequently, GTP hydrolysis enables mitofusin ubiquitylation and turnover, possibly allowing membrane approximation. Finally, MitoPLD in mammals and Ugo1 in yeast facilitate the last membrane merging step in OM fusion, resulting in fused mitochondrial OMs. The arrows indicate the conformational changes at the hinge regions based on the different conformations observed with BDLP [158].

[128]. Moreover, previous in vivo observations revealed that overexpression of mitochondrial bound C-terminal coiled coil caused mitochondrial aggregation or induced mitochondrial clustering [37]. However, it is currently not clear how to reconcile such an antiparallel association with the structural data from EHD2 or BDLP, where interactions between the G domains appear to be a constant feature [157,161,162].

6.1.2. GTPase properties of mitofusins—binding and hydrolysis of GTP are required at distinct steps of OM fusion

In both yeast and mammals, nucleotide binding enables mitofusins to adopt a dimeric conformation in cis, whereas GTP hydrolysis occurs only after trans associations between two mitochondria [47,48,124]. In analogy to other DRPs, GTP hydrolysis could trigger a conformational change on the GTP bound stretched arrangement observed for BDLP, but maintaining G domain associations. This would favor membrane approximation, in agreement with the short distances observed between two adjacent mitochondria in mitoPLD mutants [126].

OM fusion occurs at lower rates of GTP, but requires GTP binding and hydrolysis. In EHD2, the slow rate of nucleotide hydrolysis is correlated to extensive tubule formation [162]. Therefore, slow hydrolysis rates could favor mitofusin self-assembly over disassembly, promoting membrane tubulation and mediating lipid bilayer destabilization, followed by fusion. Alternatively, in Atlastins it was shown that GTP hydrolysis occurs after ER membrane fusion, making it possible that mitochondria also have such a post-fusion GTP hydrolysis role that would favor mitofusin disassembly and recycling [163].

6.1.3. Ubiquitylation of Fzo1 as a positive drive for mitochondrial fusion

It was shown that the SCF^Mdm30 E3 ligase controls ubiquitylation of the yeast mitofusin [124], at late stages during the fusion cycle, given that GTP hydrolysis is required for this post-translational modification [47,123,124]. Compatible with the aforementioned GTP hydrolysis-induced conformational change of mitofusins, the interaction of Mdm30 with the GTPase domain of Fzo1 was inhibited in full-length GTPase mutants [124]. In addition, this interaction was restored after deletion of the C-terminal domain of Fzo1, compatible with a closed and static conformation in GTP binding mutants. As Mdm30 also mediates Fzo1 turnover [111], several laboratories proposed that ubiquitylation and clearance of mitofusins would, at a late stage, facilitate close membrane approximation, necessary for completion of fusion [47,123,124]. Interestingly, two arguments suggest that Mdm30 could regulate Fzo1 turnover in a non-classical manner: overexpression of Mdm30 lacking the F-box motif partially complemented the turnover of Fzo1 [112] and completely rescued the morphologic defects of Δmdm30 cells [121]. Consistent with this role for yeast mitofusin turnover in OM fusion, the BDLP homolog from Bacillus subtilis, which can tether apposing membranes, led to membrane fusion upon protease addition [164].

6.1.4. Mfn1 and Mfn2—redundant yet different

The existence of two mitofusins in mammalian cells, Mfn1 and Mfn2, raises the question of their individual roles in OM fusion. Only simultaneous deletion of Mfn1 and Mfn2 completely inhibits mitochondrial OM fusion, clearly demonstrating their redundant nature. Consistently, overexpression of Mfn2 in Mfn1 deficient cells was sufficient to rescue mitochondrial morphology and vice versa, although to a lower extent [4]. In fact, Mfn1 and Mfn2 form both homotypic and heterotypic complexes [4,40]. In addition, the C-terminal coiled coil of Mfn1 physically interacts with both Mfn1 and Mfn2 [128].

Although there are redundancies, both Mfn1 and Mfn2 are required for optimal OM fusion levels in vitro, suggesting that each may have specific functions [25]. They do show exclusive roles and KO of Mfn1 or Mfn2 results in different mitochondrial morphologies [4]. Absence of Mfn1 in mammals, and also Fzo1 mutants in yeast,

presents numerous small mitochondria scattered throughout the cytoplasm [4,26,27,40]. Loss of Mfn2 in mammals also leads to mitochondrial fragmentation, however the fragments are larger, heterogeneous in size, and sometimes accumulate perinuclearly [4,40]. Depletion of both Mfn1 and Mfn2 gives rise to a mitochondrial morphology resembling depletion of Mfn1 alone [40]. Moreover, after Mfn1 depletion intermixing of two non-fused mitochondrial populations is unaffected [40]. In contrast, no intermixing was detected following Mfn2 depletion, suggesting that Mfn2 is involved in the cellular positioning of mitochondria [40].

Attesting for the differences between both mitofusins is the fact that mutations in Mfn2 but not in Mfn1 cause the Charcot–Marie–Tooth type 2A (CMT2A) neuropathy. Moreover, mitochondrial fusion or transport defects in CMT2A mutant cell lines can be complemented by overexpressing Mfn1 but not Mfn2 [165,166]. In fact, Mfn1 and Mfn2 are expressed differently in different tissues, being Mfn1 expression higher in more tissues than Mfn2 [39,40]. However, Mfn2 is the prevalent species in heart, skeletal muscle and brain [39,40]. This provides a possible explanation for the neurodegenerative disorders and cardiopathies specifically associated with Mfn2. Obesity or diabetes lead to a reduced expression of Mfn2 [42,167,168] and Mfn2 has been related to several cellular functions such as oxidative metabolism, cell cycle, cell death and mitochondrial axonal transport [169]. Interestingly, Mfn2 binds to Miro, an adaptor to the kinesin motor proteins and to microtubules [166]. Consistently, Mfn2 depletion inhibits axonal transport of mitochondria or proper mitochondrial positioning within neurons [166,170,171]. This could account for the axonal survival dependence on mitochondrial fusion, contributing to the neurological phenotypes associated with Mfn2 mutations.

Several other differences have been observed between Mfn1 and Mfn2, further suggesting that each Mfn protein has specialized functions, and this both within and outside OM fusion. For example, Mfn2 was suggested to play a role in tethering between mitochondria and the ER [172] and OPA1 was shown to require Mfn1 but not Mfn2 to induce mitochondrial tubulation [90]. Moreover, in vitro analyses showed that Mfn1 mediates mitochondrial tethering more efficiently than Mfn2 [48]. Consistently, the oligomerization changes observed for Fzo1 and Mfn1 upon tethering were not obvious for the Mfn2 protein [47,48]. However, cells expressing only Mfn1 fused in vitro to cells expressing only Mfn2, suggesting that trans associations between Mfn2 and Mfn1 are sufficient to drive OM fusion [25]. The situation was different in cellular culture, because Mfn1 depleted cells did not fuse with Mfn2 depleted cells [46]. Finally, Mfn1 and Mfn2 appear to have different GTP binding and hydrolysis properties [48]. Mfn2 resembles more small regulatory signaling GTPases like Ras, with high GTP binding and low GTP hydrolysis, whereas Mfn1 and Fzo1 resemble the mechanical DRPs. Along this line, a signaling function has been proposed for human Mfn2, because a mutant form of Mfn2 constitutively GTP-bound was functional and capable of accelerating fusion [173]. However, it would be interesting to test a similar constitutively GTP-bound mutation in Mfn1. Based on these observations we may speculate that Mfn1 would be primarily involved in mitochondrial tethering, more similar to the classical DRPs, whereas Mfn2 would mainly assume a regulatory role, closer to the Ras-like regulatory GTPases. However, how these two proteins cooperate to co-ordinate mitochondrial fusion is not yet clear. Consistent with this concept is the proposed positive regulatory role of Bax in mitochondrial fusion exclusively via Mfn2. The idea that Mfn2 could act after mitochondrial tethering is supported by the clustering but fusion impairment observed after depletion or KO of Mfn2 [4,40].

6.2. IM fusion model

It is now clear that IM fusion requires an initial and conserved step involving partial cleavage of the long to short isoform(s) of Mgm1/OPA1 [77,78]. In addition, and in analogy to mitofusins, these DRPs

promote tethering of the two apposing IM [22], which can establish direct contacts once OM fusion has occurred. Although it was shown that both homo and hetero-oligomers of long and short forms exist [91,92,156], precisions on the oligomeric order and composition that promotes fusion are not yet known. Interestingly, proteolytic processing stimulates GTPase activity providing a rational basis for the requirement of a combination of both short and long isoforms [92]. It is possible that the long forms contribute in membrane tethering, whereas the short forms contribute with GTPase activity [92]. As expected, both phospholipid interaction and oligomerization are required for stimulated GTPase activity [92,174]. These findings, further detailed below, applied to modeling of splice variant 7 of OPA1 to the structure of human dynamin [159,160] allow one to propose the model depicted in Fig. 3.

6.2.1. Partial proteolytic processing of Mgm1/OPA1 is required for IM fusion

The first step in allowing IM fusion is probably the constitutive partial cleavage of the responsible DRPs to allow the equimolar combination of both short and long isoforms, as referred above. Clearly, both isoforms have different properties in terms of membrane association and GTPase activity, and therefore should play distinct roles in IM fusion, as discussed below. In addition, the submitochondrial distribution of the long and short isoforms of Mgm1 between inner boundary membrane and cristae was markedly different [91]. Enrichment of the long isoform in cristae, added to the fact that these forms are devoid of GTPase activity [91,92], is consistent with the proposed role of the homotypic interactions in maintaining cristae structure [22,75].

6.2.2. Oligomerization of Mgm1/OPA1 in cis and in trans

An indication that IM tethering precedes IM fusion came from the observation that some mutant alleles of Mgm1, blocked for IM fusion in vitro, could still clearly tether IM from isolated mitochondria [22]. Importantly, other mutant alleles of Mgm1 had lost the capacity for IM tethering, indicating a crucial role for the IM DRPs in IM tethering. In addition, in vitro studies showed intra-allelic complementation of inactive Mgm1 isoforms expressed in separate populations of mitochondria, suggesting that self-oligomerization in *trans* drives IM fusion. Moreover, physical interactions between Mgm1 subunits have been observed, including evidence for both homotypic and heterotypic associations of the different isoforms, i.e. long with long, short with short and long with short [91,92,156]. Therefore, it seems that IM fusion relies on direct *trans* interactions between the DRPs of each fusion partner. These physical interactions between short and long isoforms in *cis* and in *trans* indicate that together they form a functional dimer in *cis*, which constitutes the building block for
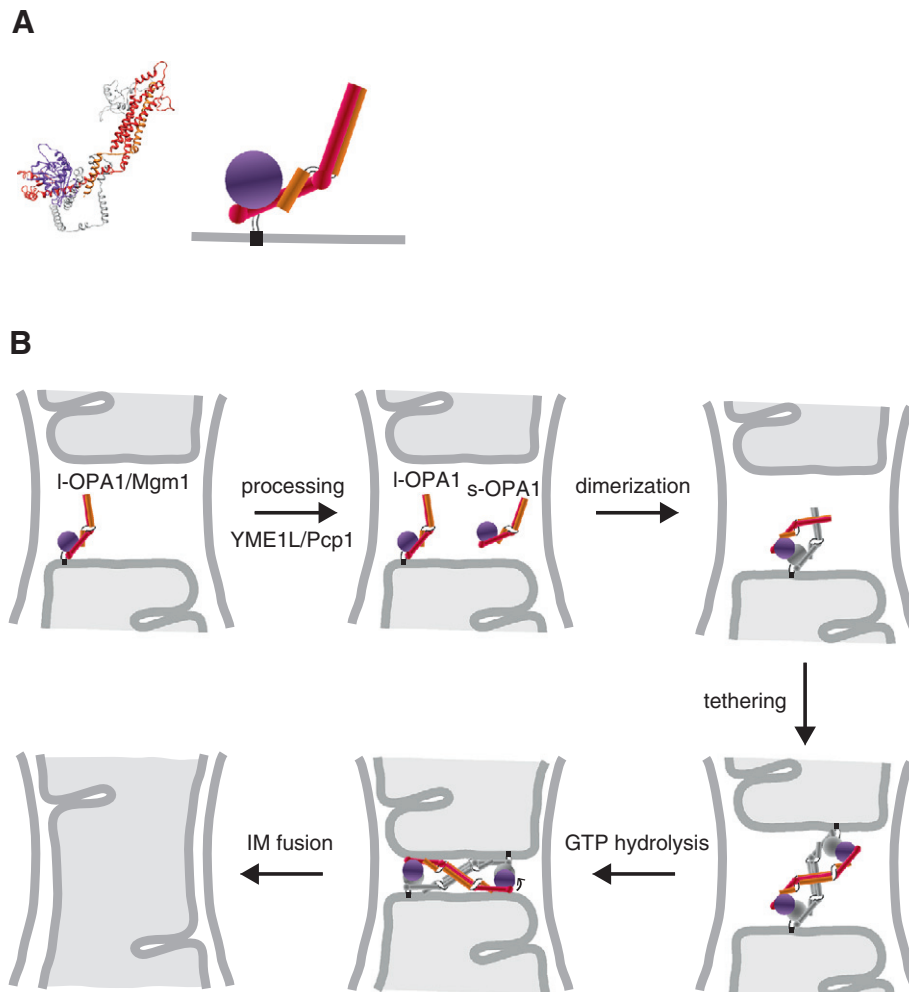
Fig. 3. IM fusion model, requiring OPA1/Mgm1 activation, oligomerization and GTP hydrolysis to achieve fusion. (A) Structural and schematic model of human OPA1. Human OPA1 (splice variant 7) was modeled on human dynamin using i-tasser (C-score −1.98) [159,160,199]. The GTPase domain is represented in purple, the helical domains are red and orange. (B) Model for mitochondrial IM fusion. L-OPA1 is anchored to the IM by an N-terminal transmembrane domain, the rest of the protein resides in the IMS. L-OPA1/Mgm1 is partially cleaved by YME1L/Pcp1, generating an equilibrium between the two isoforms, which dimerize on one membrane. IM tethering involves *trans* interactions between OPA1/Mgm1. GTP hydrolysis probably induces a conformational change possibly triggering convergence of the opposing membranes before IM fusion. The arrow indicates the conformational change at the hinge region upon GTP hydrolysis based on dynamin [159,160].

higher-order assemblies in *trans* [92]. This reveals that Mgm1 possesses one interface to form dimers and a second dimer–dimer interface for the assembly of the higher order structures. In analogy to the fission dynamins, it is formally possible that OPA1/Mgm1 promotes highly curved and fusogenic ends, by *cis* associations to higher order complexes, promoting membrane budding or tubulation. However, tubulation was not yet observed and instead ordered lattices were obtained [92,174]. Although the precise nature and order of the association into a complex of Mgm1/OPA1 have not been reported, it was proposed that stacked trimers of s-Mgm1 on apposing membranes would facilitate fusion [174].

6.2.3. GTPase activity requires processing of the IM DRP

It was shown that purified short forms of Mgm1 possess GTPase activity, self-assemble into low-order oligomers and bind to negatively charged phospholipids [156]. Moreover, binding to lipids by the short isoform is required for proper membrane fusion [174]. Consistently, after purification and reconstitution in liposomes mimicking the IM composition, short Mgm1 isoforms acquire GTPase activity in a cardiolipin-dependent manner [92]. Moreover, GTP binding or hydrolysis seems to lead to conformational changes on short Mgm1, indicated by different arrangements on the liposomes in the presence or absence of GTP. Similarly, in the presence of liposomes mimicking the IM composition, l-Mgm1 integrated in the membranes apparently with the correct topology. However, in contrast to s-Mgm1, l-Mgm1 failed in acquiring GTPase activity, possibly because of the sterical hindrance on GTP hydrolysis by the close proximity of the GTPase motif to the membrane. 3D reconstruction of purified s-Mgm1 in the presence of CL revealed the formation of the dimeric building blocks.

Just like in the OM, IM fusion is abolished in GTPase mutants of Mgm1/OPA1 [89]. This phenotype allowed one to prove that GTPase activity is only required in the short isoforms of the DRP [91,92]. However, maximal GTPase activity requires the additional presence of long isoforms [92]. Therefore, association of the long isoforms with the short isoforms appears to activate the GTPase domain of the short isoforms.

7. Inhibition of mitochondrial fusion by inactivation of the key fusion mediators

In contrast to the proteolytic processes discussed above, i.e. constitutive proteolysis of mitofusins or the partial processing of Mgm1/OPA1, which are required for mitochondrial fusion, anti-fusion proteolysis leading to mitochondrial fragmentation also occurs. In fact, a connection between mitochondrial morphology and the energetic or metabolic status of the cell has long been observed [168]. For example, dissipation of membrane potential leads to fragmentation of mitochondrial tubules [153]. This mechanism plays a quality control and protective role, where non-functional mitochondria that lose membrane potential are rendered fusion incompetent and fragment by ongoing fission. Therefore, these damaged mitochondria cannot mix to and injure the healthy population and become substrates for the autophagic pathway [132]. Also the proliferative status impinges on mitochondrial morphology: in most quiescent cells mitochondria fragment and mitochondrial tubules are only restored after growth resumption. This constitutes an essential prerequisite to entry into S phase [175]. Interestingly, in both cases, i.e. mitochondrial dysfunction and transition to quiescence, it was shown that fragmentation is accompanied by inactivation of the two key mediators of mitochondrial fusion. Upon entry into quiescence, it was recently shown that the APC/C[CDH1] E3 ligase triggers ubiquitylation of Mfn1 and OPA1, recognized as a sign for their proteasomal degradation [176]. This APC-dependent mechanism could be conserved, as in yeast it was also shown that mitochondrial fragmentation induced by cellular growth arrest is accompanied by proteasomal degradation of Fzo1 [177]. Finally, mitochondrial dysfunction leads to ubiquitylation and turnover of mitofusins and also triggers OPA1 inactivation by elimination of its long isoforms, as further detailed below (Fig. 4).

7.1. OM fusion inactivation—ubiquitylation of mitofusins as a mark for destruction

Post-translational modifications modulate a vast number of cellular events, one example being ubiquitylation of a protein as a signaling mark for its destruction. It is clear that ubiquitylation plays a central and conserved role in mitochondrial dynamics regulation. As stated above, two opposite outcomes arise from ubiquitylation of mitofusins: decreased or increased OM fusion, i.e. anti- or pro-fusion. Pro-fusion ubiquitylation of the yeast mitofusin, which is associated with the fusion cycle, depends on active GTP binding and hydrolysis [47,124]. However, the GTPase dependence for mitofusin anti-fusion ubiquitylation and destruction after membrane depolarization has not been investigated so far. Whereas the pro-fusion role is played by the SCF[Mdm30] in yeast [112], other ubiquitin ligases like the above mentioned APC/C[Cdh1] [176], the recently identified Huwe1 [178], and Parkin, mediate the anti-fusion ubiquitylation of mitofusins, in both flies and mammalian cells [14,179–184]. Huwe1 was shown to be recruited to the OM upon several apoptotic stimuli, resulting in mitochondrial fragmentation. Interestingly, this implied phosphorylation of Mfn2 by JNK and subsequent ubiquitylation and proteasomal degradation of Mfn2 [178].

Upon dissipation of the membrane potential by CCCP addition, Parkin accumulates at the OM surface of mitochondria, which results in elimination of dysfunctional mitochondria [185–187]. However, it is still unknown if more subtle mitochondrial dysfunctions, more prone to occur naturally, such as in the context of mitochondrial disease, are sufficient to accumulate Parkin and trigger the activation of mitophagy.
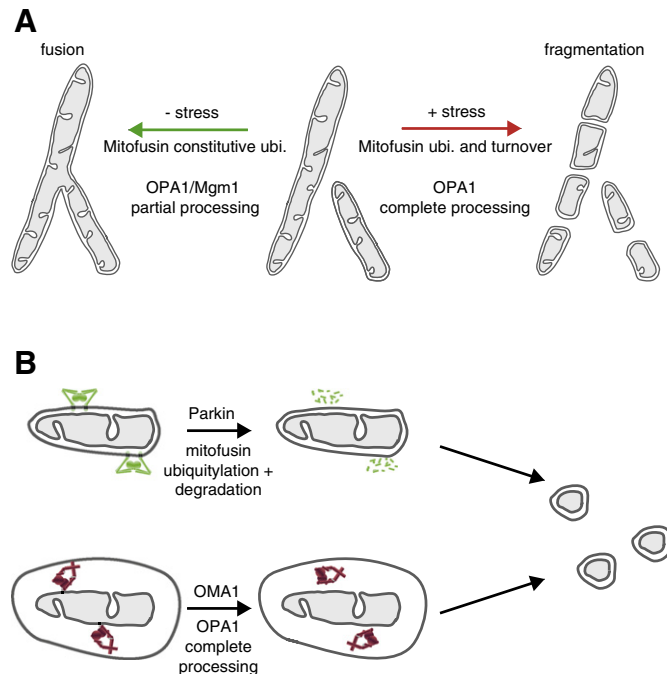
Fig. 4. Stress induced fragmentation of mitochondrial fusion. (A) Opposing consequences of mitofusin ubiquitylation and OPA1/Mgm1 processing on mitochondrial morphology. Ubiquitylation of mitofusins and partial processing of OPA1/Mgm1 are constitutively required for mitochondrial fusion (− stress). In contrast, cellular stress (+ stress) results in mitochondrial fragmentation, which also involves ubiquitylation of mitofusins and processing of OPA1. (B) OM and IM inhibition of mitochondrial fusion by Parkin and OMA1, respectively. Upon stress Parkin ubiquitylates mitofusins (upper part), thereby mediating their proteasomal degradation and OMA1 completely processes OPA1 isoforms (lower part). This results in inhibition of mitochondrial fusion and leads to a fragmented morphology due to ongoing fission.

Alternatively, this could represent a pathway dedicated to completely depolarized mitochondria. Interestingly, upon mitochondrial depolarization Parkin binds to Miro/Milton, and leads to its proteasomal dependent turnover [188]. This results in detachment of kinesin from mitochondria, thereby blocking organelle motility, which may segregate damaged mitochondria to allow their efficient clearance.

In addition to mitofusins, several other substrates ubiquitylated by Parkin have been identified, which triggers their turnover by the proteasome [189,190]. Formation of K48-linked ubiquitin chains and simultaneous recruitment of the AAA-type ATPase p97/Cdc48 to the surface of mitochondria have been observed and were proposed to mediate extraction of the Parkin substrates before their turnover [191,192]. Mechanistically, little is known on the relevance of this turnover of OM proteins for PD. Moreover, it will be interesting to analyze the role of another Parkin substrate, PARIS, which mediates the loss of dopamine neurons, the hallmark of PD [193].

7.2. IM fusion inactivation—impairment of the balance between long and short OPA1/Mgm1

In addition to the constitutive pro-fusion cleavage of OPA1 at site S2, under stress conditions an additional cleavage event of the long OPA1 isoforms at site S1 occurs in mammalian cells [78,83,85,194]. As stated above, partial cleavage of OPA1 at S2 produces a combination of long and short OPA1 isoforms, required for mitochondrial fusion [78]. In contrast, complete conversion of long to short OPA1 isoforms at site S1 hampers mitochondrial fusion. Several conditions result in this stress-induced cleavage at site S1, including low ATP levels, dissipation of the membrane potential, cold- or heat-stress, induction of apoptosis or depletion of crucial mitochondrial proteins like Prohibitins or the *m*-AAA protease [78,83,85–88,194–198]. The protease performing this inducible cleavage is the ATP-independent zinc metalloprotease OMA1 [86–88]. However, it is not yet clear how stress activates OPA1 cleavage, which is also occurring in isolated mitochondria after dissipation of the membrane potential [25]. OMA1 KO mice present several metabolic dysfunctions, suggesting that this protease could be a general sensor of metabolic stress [88]. In addition, a role for PARL in OPA1 cleavage has been proposed [76,198]. However, OPA1 processing was unaffected in both mice and fly mutated or completely depleted of the PARL proteins, along with the absence of mitochondrial fragmentation [85,98].

8. Concluding remarks

Mitochondrial membrane fusion has progressed from the genetic identification and biochemical characterization of the relevant proteins, i.e. determination of their associations and topologies, to the current era of mechanistic studies. Clearly, a major challenge for the future is to study each of the proteins identified using reconstituted systems like proteoliposomes. This will not only enhance the understanding of the role of each component so far identified but also allow one to further stage the molecular mechanisms required for fusion of each of the mitochondrial membranes. Moreover, it is getting increasingly clear that mitochondria function as signaling platforms and future experiments, certainly facilitated by the recently developed automated assay for quantifying mitochondrial fusion, will allow one to shed light on the molecular implications of mitochondrial fusion in disease.

Acknowledgements

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