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# A Molecular Perspective on Mitochondrial Membrane Fusion: From the Key Players to Oligomerization and Tethering of Mitofusin

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1 **Title:**

2 Current updates on mitofusin oligomers and mitochondrial membrane fusion.

3 **A molecular perspective on mitochondrial membrane fusion: from the key**  
4 **players to oligomerization and tethering of mitofusin**

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18

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22

23 **Abstract:**

24 Mitochondria are dynamic organelles characterized by an ultrastructural organization which is  
25 essential in maintaining their quality control and ensure functional efficiency. The complex  
26 mitochondrial network is the result of the two ongoing forces of fusion and fission of inner and  
27 outer membranes. Understanding the functional details of mitochondrial dynamics is  
28 physiologically relevant as perturbations of this delicate equilibrium have critical consequences and  
29 involved in several neurological disorders. Molecular actors involved in this process are large  
30 GTPases from the dynamin-related protein family. They catalyze nucleotide-dependent membrane  
31 remodeling and are widely conserved from bacteria to higher eukaryotes. Although structural  
32 characterization of different family members has contributed to understand molecular mechanisms  
33 of mitochondrial dynamics in more detail, the complete structure of some members as well as the  
34 precise assembly of functional oligomers remain largely unknown. As increasing structural data

35 becomes available, the domain modularity across the dynamin superfamily emerged as a foundation  
36 for transfer the knowledge towards less characterized members. In this review we will first provide  
37 an overview of the main actors involved in mitochondrial dynamics. We then discuss recent  
38 example of computational methodologies for the study of mitofusin oligomers, and present how the  
39 usage of integrative modeling in conjunction with biochemical data can be an asset in progress the  
40 still challenging field of membrane dynamics.

41

42 **Keywords:**

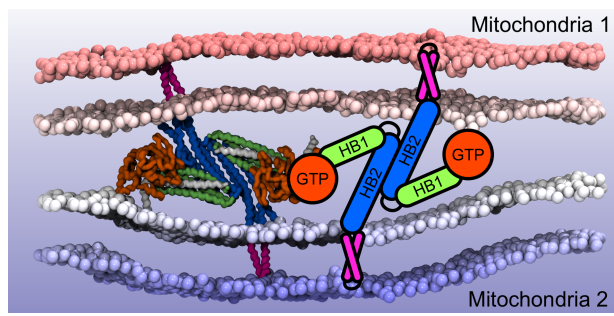
43 Mitofusin, Fzo1, mitochondrial dynamics, mitochondrial fusion, mitochondrial fission, dynamin-  
44 related proteins.

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## 49 **Introduction**

50

51 Mitochondria are central vital organelles widely involved in many important cellular functions.  
52 They possess a doubled-membrane apparatus which is inherently connected to their ultrastructural  
53 organization. Our current understanding about their morphology is beyond the initial beliefs that  
54 depicted them as simple rod-shaped bodies. They are far from being isolated and indeed very  
55 dynamic during cell life. Mitochondria continuously move on cytoskeletal tracks, divide and fuse,  
56 forming shapes that range from isolated corpuscles to filamentous networks (Braun and  
57 Westermann 2011, Chan 2012, Cohen and Tareste, 2018, Tilokani et al., 2018). This precisely  
58 controlled process is known as mitochondrial dynamics for which they evolved a dedicated set of  
59 protein machineries (Friedman and Nunnari. 2014, Mattie et al., 2019). When particular cell  
60 locations are in need of high ATP production, mitochondria are fragmented and mobilized in these  
61 sites (Skulachev 2001; Li et al. 2004; Kuznetsov et al. 2009). Similarly, an efficient organellar  
62 fragmentation also ensures propagation of mitochondrial genome from mother to daughter cell  
63 (Taguchi et al. 2007; Altmann et al. 2008; Ishihara et al. 2009). Isolation of damaged or senescent  
64 mitochondria from the network followed by degradation through a specific form of autophagy (i.e.  
65 mitophagy), ensures proper quality control of the mitochondrial network (Khaminets et al. 2016;  
66 Georgakopoulos et al. 2017). Nevertheless, too much fission lead to mitochondrial heterogeneity  
67 and dysfunction in yeast as well as in mammalian cells (Chen et al. 2005, 2007; Merz and  
68 Westermann 2009). Because of that, a concerted fusion activity counterbalances potential excessive  
69 fragmentation. Healthy mitochondria fuse back to the existing network which homogenizes their  
70 proteomic content. Fusion also contributes to face the gradual accumulation of mutations in the  
71 nucleoid (i.e. mtDNA) through genetic complementation (Ono et al. 2001; Balaban et al. 2005;  
72 Maiese 2016). Therefore, fusion and fission processes are critical steps of cellular quality control  
73 through which mitochondria maintain their homeostasis (Itoh et al. 2013; Liesa and Shirihai 2013).  
74 Perturbations of this delicate equilibrium are associated with several diseases which are mostly  
75 neurological disorders (Bertholet et al. 2016). This is due to the fact that neuronal cells are  
76 energetically demanding and thus particularly sensitive to mitochondrial dynamics (Knott et al.  
77 2008). Therefore, understanding the mechanistic details of mitochondrial dynamics is  
78 physiologically and medically relevant.

79

80 The main factors mediating mitochondrial dynamics are multi-domain mechano-chemical GTPases  
81 that belong to the dynamin superfamily (Praefcke and McMahon 2004; Ferguson and De Camilli

82 2012; Daumke and Praefcke 2016). These Dynamin-Related Proteins (DRPs) are mostly implicated  
83 in membrane-remodeling processes (Daumke and Praefcke 2016; Ramachandran 2018).

84

85 Human Drp1 (Dnm1 in yeast) mediates the scission of mitochondria (Smirnova et al. 2001; Kraus  
86 and Ryan 2017). The role of Drp1 appears to be highly conserved in eukaryotes (Labrousse et al.  
87 1999; Sesaki and Jensen 1999; Smirnova et al. 2001; Aldridge et al. 2007; Logan 2010) and cells  
88 lacking Drp1 (or Dnm1) contain highly interconnected mitochondria networks (Otsuga et al. 1998;  
89 Smirnova et al. 1998). Drp1 is recruited to the mitochondrial outer membrane (OM) by protein  
90 adaptors: the mitochondria fission factor (Mff) and the two mitochondrial dynamics proteins MiD49  
91 and MiD51 (Otera et al. 2010; Palmer et al. 2011). Investigation into individual and combined loss  
92 of each adapter showed a combined prominent role of MiD51/Mff (Osellame et al. 2016). In yeast,  
93 Dnm1 localization relies on the integral OM protein Fis1 and two adaptors Mdv1 and Caf4 (Mozdy  
94 et al. 2000; Tieu et al. 2002; Okamoto and Shaw 2005; Griffin et al. 2005) which recruit Dnm1 to  
95 mitochondria. Fis1 is conserved in human (Dohm et al. 2004) but its function evolutionary diverged  
96 to act as an inhibitor of the mitochondrial fusion machinery (Yu et al. 2019). After Drp1/Dnm1 is  
97 recruited to the mitochondrial OM (Sesaki and Jensen 1999; Smirnova et al. 2001) its innate  
98 capacity to assemble into multimeric complexes, leads to membrane constriction upon GTP  
99 hydrolysis (Mears et al. 2011; Fröhlich et al. 2013). Rings and spirals-like structures of Drp1  
100 assemble around the organelle at region of contact with the endoplasmic reticulum (ER), which is a  
101 requisite for the fission event (Friedman et al. 2011). ER tubules wrap around the organelle to  
102 decrease its average diameter from 300-500 nm to 150 nm (Friedman et al. 2011). Another  
103 Dynamin, Dyn2, was suggested to collaborate with Drp1 (Lee et al. 2016), but the involvement of  
104 this second DRP in mitochondrial division is controversial (Fonseca et al. 2019).

105

106 OPA1(optic atrophy 1) and its yeast homologue Mgm1 (mitochondrial genome maintenance 1)  
107 mediate the fusion of the mitochondrial inner membrane (IM) and participate in cristae organization  
108 (Wong et al. 2000; Frezza et al. 2006). The fusion of the mitochondrial OM is triggered by the  
109 mitofusins Mfn1 and Mfn2 in mammals and by the fuzzy onion Fzo1 protein in yeast (Hales and  
110 Fuller 1997; Cohen and Tareste 2018). Notably, the phylogenetically related group of atlastin (and  
111 its yeast homologue Sey1p), functions in fusion of ER tubules (Hu et al. 2009; Orso et al. 2009).  
112 Several structural observations on recombinant atlastins and Sey1 have provided deep insights into  
113 fusion of ER tubules (Yan et al., 2015, Kim et al., 2017, Lee et al., 2019). In contrast, fulllength  
114 mitofusins have not been purified which has limited the study of their assembly and

115 oligomerization, which is an essential step in the OM fusion process (Rapaport et al. 1998; Fritz et  
116 al. 2001; Ishihara et al. 2003, 2004; Santel et al. 2003; Koshiba et al. 2004; Brandt et al. 2017).

117

118 Integration of physics-based computational methodologies in the study of membrane proteins can  
119 be of great advantages to overcome current limitations in their isolation and purification. Clear  
120 examples are widely discussed in recent review works (Friedman et al. 2018; Marrink et al. 2019;  
121 Corradi et al. 2019). The involvement of mitofusins in mitochondrial fusion was also recently  
122 reviewed (Cohen and Tareste 2018). Here, we thus review and discuss recent observations on  
123 mitofusin oligomerization and dynamics in their native membrane environment based on  
124 computational approaches such as molecular modeling and molecular dynamics (MD) simulation.

125

### 126 **Mitochondrial fusion machineries**

127

128 The first known mediator of mitochondrial fusion was identified in 1997 by genetic analysis of the  
129 male sterile fuzzy onions (*fzo*) mutant in *Drosophila melanogaster* (Hales and Fuller 1997). In fruit-  
130 fly, *fzo* is a developmentally regulated gene, fundamental in the early step of spermatogenesis  
131 (Hales and Fuller 1997). Its paralogue protein, Marf/Dmfn, was later found to be ubiquitously  
132 expressed (Hwa et al. 2002). The discovery of *fzo* lead to subsequent identification of the conserved  
133 protein family that was termed mitofusin in human and fuzzy onions in yeast (Hales and Fuller  
134 1997; Rapaport et al. 1998; Hermann et al. 1998; Rojo et al. 2002; Logan 2010). Mitochondria  
135 evolved two independent machineries involved in the fusion process of their two independent  
136 membranes. The mitofusins/*fzo* machinery is responsible for the OM fusion, whereas OPA1/Mgm1  
137 deliver IM fusion. Mutant cells for these families of proteins contain fragmented mitochondria in  
138 yeast (Rapaport et al. 1998; Hermann et al. 1998; Wong et al. 2000) and mammals (Chen et al.  
139 2003; Olichon et al. 2003), furthermore, fused cells lacking mitofusins, Mgm1 or OPA1 cannot mix  
140 their mitochondrial matrix contents, indicating a lack of fusion (Hermann et al. 1998; Wong et al.  
141 2000; Chen et al. 2003; Cipolat et al. 2004). Mitofusins are expressed as a single copy in  
142 invertebrates and lower eukaryotes, such as the yeast *Fzo1* (Rapaport et al. 1998; Hermann et al.  
143 1998), while vertebrates express two paralogues, mitofusin *Mfn1* and *Mfn2* with high sequence  
144 similarity (Rojo et al. 2002).

145

146 The second member of the DRP family required for mitochondrial IM fusion is *Opa1/Mgm1*  
147 (Cipolat et al. 2004; Meeusen et al. 2006; Griparic et al. 2007; Kanazawa et al. 2008). They localize  
148 in the mitochondrial inter-membrane space (IMS) and are targeted to the IM thanks to an N-

149 terminal mitochondrial import sequence (MIS) and transmembrane (TM) helices before the GTPase  
150 domain (Wong et al. 2000; Olichon et al. 2002; Guillou et al. 2005). OPA1/Mgm1 are synthesized  
151 as precursors that undergo proteolytic processing (Herlan et al. 2003; Leroy et al. 2010). Two  
152 membrane-bound proteases, Oma1 (Ehse et al. 2009; Head et al. 2009) and Yme11 (Griparic et al.  
153 2007; Song et al. 2007), process Opa1 resulting in fragments with different molecular weights and  
154 referred as “long” (L-OPA1) and “short” (S-OPA1). An initial study suggested that the two forms  
155 alone have little fusion activity and are both required for mitochondrial fusion (Song et al. 2007).  
156 However, it is now known that L-OPA1 is sufficient to deliver fusion (Tondera et al. 2009; Anand et  
157 al. 2014; Ban et al. 2017) and that there is a precise balance between the activity of both proteases  
158 Oma1 and Yme11, though the exact role of S-OPA1 is not completely understood (MacVicar and  
159 Langer 2016). Recently a study suggested that S-OPA1 facilitates the binding of L-OPA1 to the  
160 negative charged lipid cardiolipin (CL), promoting fusion (Ban et al. 2017). This model also  
161 corroborates studies performed in yeast suggesting the recruitment of both L-OPA1 and S-OPA1  
162 isoforms as well as CL for IM fusion (DeVay et al. 2009; Rujiviphat et al. 2009). Interestingly,  
163 OPA1-dependent fusion depends on Mfn1 but not Mfn2 (Cipolat et al. 2004; Tondera et al. 2009).  
164 Besides, a specific region that confers mitofusin isoform-specific function has been identified (Sloat  
165 et al., 2019). This region provides a specific fusion activity to Mfn1 and Mfn2 and may be involved  
166 in higher order oligomerization. A functional connection between factors dedicated to OM and IM  
167 fusion is not surprising since the processes are coordinated. This observation is possibly  
168 corroborated by a recent new topology proposed for the human mitofusins according to which the  
169 C-terminal end is exposed to the mitochondrial IMS (Mattie et al. 2018). Nonetheless, other  
170 proteins such as the yeast factor Ugo1 have been identified having a possible role at the interface  
171 between the OM and IM.

172

173 Ugo1 is a 58 kDa protein embedded in the OM with the N-terminal end facing the cytosol and the  
174 C-terminal facing the IMS (Sesaki and Jensen 2001). Ugo1 was proposed to coordinate OM and IM  
175 fusion due to its ability to interact both with Fzo1 and Mgm1 (Wong et al. 2003; Sesaki and Jensen  
176 2004) creating a scaffold for the assembly of a fusion complex. The involvement of Ugo1 in both  
177 outer and inner membrane fusion was then further demonstrated (Hoppins et al. 2009). Even  
178 though no Ugo1 equivalent protein has been identified in mammals, an Ugo1-like protein, named  
179 SLC25A46, has been described in humans and shown to be present in mouse, zebrafish, fruit-fly  
180 and nematode (Abrams et al. 2015). Interestingly SLC25A46, like Ugo1, is recruited to the OM  
181 (Haitina et al. 2006). However, there is insufficient evidence to determine orthology and the protein  
182 fails to complement Ugo1 deletion in yeast (Abrams et al. 2015). As previously discussed (Cohen

183 and Tareste 2018) the exposure of the human mitofusin to the IMS (Mattie et al., 2018) could  
184 possibly compensate the lack of Ugo1 in human.

185

### 186 **Membrane fusion catalysts from bacterial homologues**

187

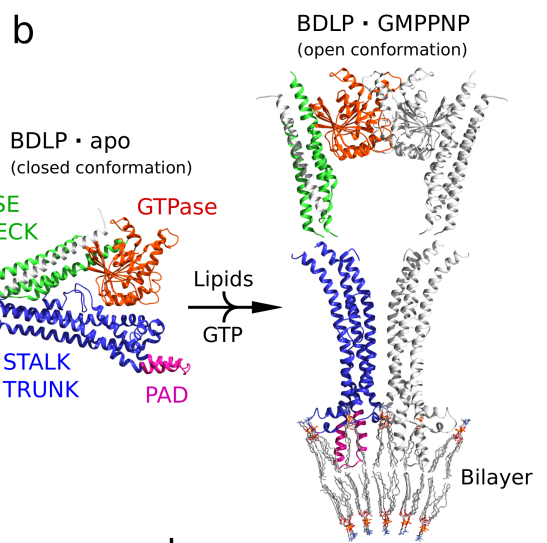
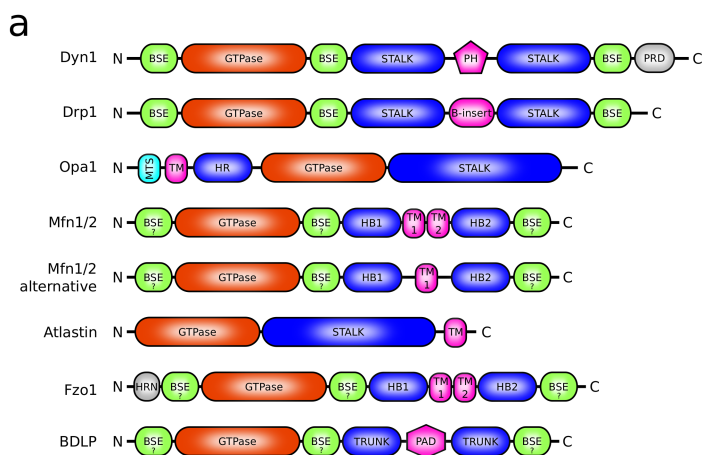
188 Interestingly, DRPs were also identified in bacteria (van der Bliëk 1999; Leipe et al. 2002; Low and  
189 Löwe 2006), plants (Fujimoto et al. 2010; Huang et al. 2015; Aung et al. 2012; Gao et al. 2003; Gao  
190 et al. 2006) and green algae (Findinier et al. 2019). They are thought to regulate cell shape and  
191 thylakoid morphology in cyanobacteria (Low and Löwe 2006; Bürmann et al. 2011) and chloroplast  
192 fragmentation or thylakoid fusion in plants (Gao et al. 2003; Gao et al. 2006; Findinier et al. 2019).  
193 Furthermore, the dynamin-like protein LeoA from a pathogenic *Escherichia coli* strain has been  
194 assigned a role in the secretion of bacterial vesicles for enhancing the release of toxins (Michie et al.  
195 2014). Although the function of bacterial DRPs/DLPs was largely unknown, *in vitro* analyses on  
196 DynA from *Bacillus subtilis* revealed its capacity of tethering and merging membranes, suggesting  
197 a possible role at the site of septation during the cytokinesis phase (Bürmann et al. 2011). Moreover,  
198 DynA shared also the ability to seal membrane gaps originated by membrane stress, thus  
199 contributing to the innate immunity of bacteria (Sawant et al., 2016). Many bacterial species contain  
200 more than one dynamin gene organized in an operon with two of them often found in tandem  
201 (Bürmann et al. 2011, Boot et al., 2017, PMID: 29281637, Boot et al., 2016, PMID: [27474746](#)).  
202 Some species even harbour a fusion of the two genes incorporating two GTPase domains, like the  
203 DynA protein in *B. subtilis*. In this latter case, the protein is not cleaved into separate subunits and is  
204 able to tether two membranes. Among the two parts, only the subunit called D1 was found to be  
205 associated with the membrane when expressed in a unique construct with the green fluorescent  
206 protein (Bürmann et al. 2011). The subunit D1 can not only bind to the membrane, but also create  
207 an extensive tethered zone of clustered liposomes and promote GTP-independent membrane fusion  
208 (Bürmann et al. 2011). Interestingly, the process was observed to depend on magnesium ions, which  
209 facilitate also membrane fusion in areas of high membrane curvature and proximity (Wilschut et al.  
210 1981). Therefore, a role as passive catalysts has been proposed and the GTP hydrolysis might not be  
211 needed to directly energize the reaction, but regulate whether and how the dynamin complex enters  
212 a fusogenic-state (Bürmann et al. 2011).

213 The BDLP (bacterial dynamin-like protein), a mitofusin homologue from the filamentous  
214 cyanobacteria *Nostoc punctiforme*, was shown to induce GTP-dependent deformation and  
215 tubulation of membranes (Low et al. 2009). Although BDLP is soluble in water (Low and Löwe  
216 2006), it includes a membrane binding domain called *paddle* and mutations located in this region

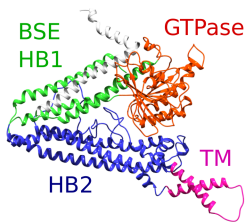
217 abrogate lipid binding (Low and Löwe 2006). The BDLP apoenzyme and GDP-bound crystal  
218 structures reveal a compact molecule comprising a GTPase domain which is in close contact with  
219 two helical bundles, called *neck* and *trunk* (PDB: 2J69) (Low and Löwe 2006) (Fig. 1b). BDLP  
220 tubulates *E. coli* liposomes in presence of a non-hydrolysable GTP analogue (Low et al. 2009).  
221 Under these conditions, BDLP forms coated tubes that resemble those formed by the eukaryotic  
222 dynamin 1 in the presence of phosphatidylserine liposomes (Sweitzer and Hinshaw 1998; Daumke  
223 and Praefcke 2016). In both cases, an “open” molecule forms an extended repeat that represents the  
224 basic assembly unit of a helical filament (PDB: 2W6D) (Low et al. 2009). Indeed the BDLP cryo-  
225 EM reconstruction is compatible with the interpretation that BDLP mediates lipid curvature through  
226 a long-range conformational change between a “closed” and an “open” state (Low et al. 2009) (Fig.  
227 1b). In BDLP, the GTPase-anchored chunk, called *neck*, moves away from the membrane-anchored  
228 *trunk* of  $\sim 135^\circ$  along the proposed hinges 1a and 1b. The entire GTPase domain also turns  $\sim 70^\circ$   
229 around two other hinge regions, 2a and 2b (Low et al. 2009). Striking evidence of bending in this  
230 region comes from the dynamin 1 crystal structures (PDB: 5D3Q (Anand et al. 2016) and 3ZYC  
231 (Chappie et al. 2011)), with a conserved proline mediating a sharp kink of  $70^\circ$ , proposed to guide a  
232 *powerstroke* reaction during membrane remodeling (Chappie et al. 2011; Daumke and Praefcke  
233 2016).

234

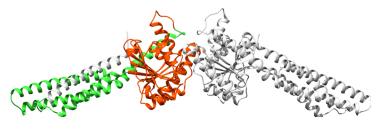
235 **Fig. 1** Schematic representation of the modular domain architecture of selected proteins from the  
236 dynamin superfamily. (a) Cartoon summarizing the modular domain architecture for selected DRPs  
237 and DLPs discussed in the text. The classical nomenclature is indicated. The heptad repeats of the  
238 mitofusins/Fzo1 (HRN, HR1 and HR2) are also called HB1 and HB2 (Helical Bundles). They are  
239 aligned to the *stalk/neck* domains of other DLPs and dynamins showing a similar architecture and  
240 they might serve as BSE (Bundle Signaling Element). MTS (Mitochondrial Targeting Sequence),  
241 TM (transmembrane), PAD (paddle), PH (Pleckstrin Homology), PRD (Proline-Rich Domain). (b)  
242 The BDLP conformational change upon GTP and lipid binding. The structure is closed in its GDP-  
243 bound (not shown) and in its apo-form (left) (PDB: 2J68 (Low and Löwe 2006)). Lipid binding  
244 concomitant with GTP hydrolysis, reveals a conformational change detected using cryo-electron  
245 microscopy (right) (PDB: 2W6D (Low et al. 2009)). (c) (Left) The Fzo1 structural model (De  
246 Vecchis et al. 2017) with HB1 and HB2. (Center) Crystal structure of the human Mfn1 fragment-  
247 construct obtained upon addition of GDP/AlF<sub>4</sub><sup>-</sup> (not shown) (PDB: 5GOM (Cao et al. 2017)) and  
248 observed in presence of GDP/BeF<sub>3</sub><sup>-</sup> (not shown) (PDB: 5YEW (Yan et al. 2018)) (Right). (d) Cryo-  
249 electron microscopy of the human dynamin 1 (Dyn1) in complex with GMPPCP (not shown) (PDB:  
250 6DLU (Kong et al. 2018)). The PRD was not present in the reconstruction



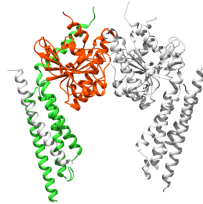
**C** Fzo1 structural model



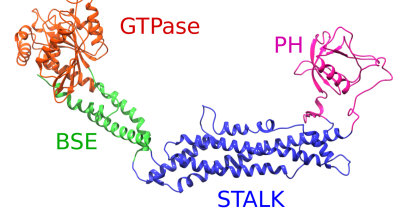
Mfn1 • GDP/AlF<sub>4</sub><sup>-</sup>



Mfn1 • GDP/BeF<sub>3</sub><sup>-</sup>



**d** Dyn1 • GMPPCP



251

252 In BDLP, as described for other dynamins (Ghosh et al. 2006; Chappie et al. 2010), the GTPase  
253 domain homodimerizes across the nucleotide binding pockets upon nucleotide binding (Low and  
254 Löwe 2006), resulting in the often called GG dimer (Chappie et al. 2010). However, the effect of  
255 nucleotide binding is not limited to GTPase domain homodimerization and GTP binding also  
256 induces lateral self-association between BDLP homodimers (Low et al. 2009).

257 Recent crystal structures of an Mfn1 fragment suggested a closed and open conformation (Qi et al.  
258 2016; Cao et al. 2017; Yan et al. 2018). The protein construct consists of the GTPase domain linked  
259 to a 4-helix bundle which is comparable to the bacterial DLPs' *neck*. Mfn1 was found to dimerize in  
260 presence of GTP or with transition state analogues (i.e. GDP/BeF<sub>3</sub><sup>-</sup> or GDP/AlF<sub>4</sub><sup>-</sup>). Crystals were  
261 obtained with the transition state analogues and showed two alternative configurations as for BDLP,  
262 with the canonical GG interface followed by the 4-helix bundle (called HB1) (Cao et al. 2017; Yan  
263 et al. 2018). Interestingly, one alternative is reminiscent of the BDLP open form in which the HB1  
264 bundle runs perpendicular to the GG interface (PDB: 5YEW (Yan et al. 2018)). Conversely, in the  
265 second alternative the HB1 runs parallel to the GG interface (PDB: 5GOM (Cao et al. 2017)) which  
266 is comparable to the BDLP closed form (Fig. 1c).

267 Altogether, even though structural information about full-length Mfns/Fzo1 are currently lacking,  
268 hints on how these proteins might function could be provided by the structure of bacterial DLPs  
269 homologous to mitofusins that were previously solved.

270

### 271 **Topology and domain composition of mitofusins**

272

273 Similarly to other members of the dynamin superfamily, mitofusins have a modular domain  
274 organization confirmed also by a recent evolutionary analysis (Purkanti and Thattai 2015; Sinha and  
275 Manoj 2019) (Fig. 1a). They likely contain three distinct structural domains as described for the  
276 bacterial DLPs (Low and Löwe 2006): i) A GTPase domain (or G-domain) characterized by low  
277 nucleotide affinity, that binds and hydrolyzes GTP without guanine nucleotide exchange factor at  
278 variance with most G-proteins (Dever et al. 1987; Vetter and Wittinghofer 2001; Ozturk and Kinzy  
279 2008) and two helical bundles probably involved in oligomerization and stimulation of GTPase  
280 activity ii) the HB1, or bundle signaling element (BSE) in human dynamin, or *neck* in bacterial  
281 dynamins (Low and Löwe 2006) and iii) the HB2, or *stalk* in eukaryotes, or *trunk* in bacterial  
282 dynamins (Fig. 1a). The GTPase domain mediates dimerization (Low and Löwe 2006; Low et al.  
283 2009; Chappie et al. 2010; Anton et al. 2011; Cao et al. 2017; Yan et al. 2018) and harbors four  
284 conserved motifs called G1 to G4, involved in nucleotide binding with distinct characteristics.



285 Remarkably, the G1 motif (also called P-loop), tightly wraps around the  $\beta$ -phosphate (Saraste et al.  
286 1990; Chappie et al. 2010, Cao et al. 2017; Yan et al. 2018) and the G2, G3 motifs (also called the  
287 switch regions) change conformation upon GTP hydrolysis in human dynamin (Chappie et al.  
288 2010). It has been hypothesized that the BSE domain bears possible functions in transferring the  
289 conformational information to the *trunk* region (Chappie et al. 2010), though the *stalk* domain is  
290 linked via a hinge to the GTPase domain in BDLP (Low et al. 2009). Additionally, many dynamins  
291 contain a domain for interactions with lipids. This can be a pleckstrin-homology (PH) domain as in  
292 the classical dynamins (Klein et al. 1998), a TM domain as in mitofusin/Fzo1 (De Vecchis et al.  
293 2017; Fritz et al., 2001; Rojo et al., 2002; Mattie et al. 2018) and atlastin (Hu et al. 2009; Orso et al.  
294 2009; [Betancourt-Solis](#) et al., 2018; Huang et al., 2017) or a *paddle* region for transient lipid  
295 attachment as in BDLP (Low et al. 2009) (Fig. 1a). This domain modularity across species is  
296 remarkable as taken singularly each protein may deliver completely different tasks that span from  
297 fusion to fission.

298

299 It is established that the yeast mitofusin Fzo1 spans the mitochondrial OM twice, exposing the  
300 protein N- and C-terminal to the cytosol (Fritz et al. 2001; Rojo et al. 2002). From their first  
301 identification and further biochemical analyses this was similarly confirmed for the human  
302 orthologues (Santel and Fuller 2001; Rojo et al. 2002; Koshiba et al. 2004; Huang et al. 2011).  
303 However, this observation was recently challenged for the Mfn1 and Mfn2, and further corroborated  
304 by a phylogenetic analysis (Mattie et al. 2018). The work suggested that mitofusins from vertebrates  
305 harbor a single TM domain exposing the protein C-terminal to the mitochondrial IMS.  
306 Nevertheless, the Mfn2 C-terminal end could be exposed to the cytosol as suggested by tagging and  
307 partially rescue mitochondrial fusion (Mattie et al. 2018). Therefore, it might still be possible for the  
308 human as well as other vertebrates to adopt two different topologies (Cohen and Tareste, 2018).  
309 This duality may facilitate the coordination between OM and IM during fusion and supply the  
310 function that Ugo1 has in yeast (Cohen and Tareste, 2018), perhaps in concert with the recently  
311 identified SLC25A46 (Abrams et al. 2015).

312

313 Mitofusin family members also contain two heptad repeat (HR) regions, called HR1 and HR2,  
314 situated as for Fzo1 on either side of the TM region. The HR2 is proposed to be exposed to the  
315 mitochondrial IMS for the vertebrate mitofusins (Mattie et al. 2018). The yeast Fzo1 encodes a third  
316 HR (named HRN) located at the N-terminal end with respect to the GTPase domain and the first 60  
317 N-terminal residues of Fzo1 are essential for mitochondrial respiration and thus for the function of  
318 the yeast mitofusin (De Vecchis et al. 2017). The hydrophobic HR motifs are predicted to form

319 coiled-coil structures (Eckert and Kim 2001) and are thought to play a critical role in tethering two  
320 mitochondria during a docking step before fusion (Koshiba et al. 2004; Daste et al. 2018). Such  
321 HRs are known to be central for other fusogenic machineries like viral fusion proteins (Skehel and  
322 Wiley 1998) and SNAREs (e.g. soluble N-ethylmaleimide-sensitive factor attachment protein  
323 receptor) (Ungar and Hughson 2003; Bonifacino and Glick 2004). However, the mechanism  
324 towards which the human mitofusin HR1 promotes bilayer fusion appear to be different with  
325 respect SNAREs or viral proteins (Daste et al., 2018). In particular, the human HR1 possibly act by  
326 destabilizing the lipid bilayer especially in region characterized by lipid packing defects (Daste et  
327 al., 2018). Structural data revealed that the HR2 region from Mfn1 (residues 660-735), (PDB : 1T3J  
328 (Koshiba et al. 2004)) forms a dimeric antiparallel coiled-coil that is 9.5 nm long (Koshiba et al.  
329 2004). Its formation was hypothesized to be an important step during a *trans* complex mitofusin  
330 dimer assembly, as this structure would mediate tethering of mitochondria. Furthermore, the study  
331 found that removal of the GTPase domain (resulting in the construct comprising residues 331-741),  
332 promotes an extensive aggregation of mitochondria in a HR2-dependent manner (Koshiba et al.  
333 2004). In contrast to this value of 9.5 nm observed by crystallography, electron microscopy revealed  
334 trapped densely packed mitochondria that maintain a uniform gap of ~16 nm between opposing  
335 OMs (Koshiba et al. 2004). Experimental observation of the yeast mitofusin Fzo1 lead to the final  
336 discovery of an average 8 nm distance between outer membranes during Fzo1-mediated  
337 mitochondrial tethering (Brandt et al., 2016). These discrepancies may finally suggest different  
338 mechanisms between the human and the yeast mitofusins.

339

340 Trans oligomers have also been described recently for bacterial DLPs. Two chains, DLP1 and DLP2  
341 from *Campylobacter jejuni*, form a *trans* tetramer through their helical bundles suggesting a  
342 mechanism that facilitates the tubulation and tethering of liposomes in trans (Liu et al. 2018). . In  
343 the research, structural data have been proposed to be homologous to human Mfn1 (Liu et al. 2018)  
344 and a molecular model of how bacterial DLP may tether liposome membranes is provided (Liu et al.  
345 2018).

346 In the last decades the community provided a wide-range of biochemical and structural data across  
347 several mitofusin-related DLPs/DRPs (Low and Löwe 2006; Low et al. 2009; Michie et al. 2014,  
348 Liu et al. 2018). This data, jointly with the domain modularity observed in DRPs ([Daumke and](#)  
349 [Praefcke, 2016](#); Sinha and Manoj 2019) might allow a transfer of structural and functional  
350 knowledge across the dynamin superfamily. even across different species as attempted for the yeast  
351 mitofusin Fzo1 (De Vecchis et al. 2017).

352

### 353 **An integrated modeling approach for building intact mitofusin models**

354

355 Several attempts have been made to model full-length structures of Fzo1 and human mitofusins  
356 because molecular modeling represents an alternative approach to investigate the possible  
357 architecture and dynamics of these large GTPases. Mitofusin closed and open conformations have  
358 been investigated using a mix of techniques from homology modeling to threading (Anton et al.  
359 2013). In this study, full-length Fzo1 was modeled using the BDLP-apoenzyme as structural  
360 template (PDB: 2J68 (Low and Löwe 2006)), whereas a truncated variant without the TM domain  
361 was modeled using the open conformation of BDLP (PDB: 2W6D (Low et al. 2009)). In another  
362 study, the threading method was also employed to model the human Mfn1 using BDLP as a  
363 template (Escobar-Henriques and Anton 2013). With similar approaches, a model of Mfn2 was  
364 subsequently proposed (Franco et al. 2016). In this model, HR1 and HR2 helical segments are  
365 locally unfolded and bent in the predicted hinge regions 1a and 1b. In this model the conformational  
366 change prior to tethering/fusion was proposed to consist in an unfolding process of the HR2  
367 domain. This hypothesis clearly contrasts with the conformational switch proposed for BDLP or for  
368 other DRPs resolved so far (Low and Löwe 2006; Low et al. 2009; Michie et al. 2014, Liu et al.  
369 2018). Therefore, this model made few consideration of the established importance of the mitofusin  
370 GTPase domain in mitochondrial fusion (Low et al. 2009; Anton et al. 2011; Brandt et al. 2016).  
371 Nonetheless, a short peptide (residues 367-384) designed to perturb intra-molecular HR1-HR2  
372 interactions, was found to rescue mitochondrial morphology in murine embryonic fibroblasts and  
373 cultured neurons expressing either a Mfn2 mutant or the naturally occurring human Charcot-Marie-  
374 Tooth type 2A (CMT2A) mutant (Franco et al. 2016). Besides, it would be of great advantage in the  
375 dissection of the mechanism underpinning the CMT2A, if these peptides would be compared with  
376 other available mitofusin and mitofusin-homologous structural models.

377 A further research proposed a complete model of the human Mfn2 ([Beręsewicz](#) et al., 2017;  
378 [Beręsewicz](#) et al., 2018). The group used the server I-TASSER that implement the threading  
379 approach (Roy et al., 2010), using the apo form of BDLP as a template (PDB: 2J69). The GTP  
380 molecule was subsequently docked in the GTPase binding site ([Beręsewicz](#) et al., 2017; [Beręsewicz](#)  
381 et al., 2018). Interestingly, the modeling stage was followed by a molecular dynamics simulations in  
382 a solvated lipid bilayer. Although the model resulted stable, in future research it would be  
383 interesting to extend the current simulations that were run for decades of ns ([Beręsewicz](#) et al.,  
384 2017; [Beręsewicz](#) et al., 2018).

385

386 In contrast to the mitofusin models mentioned above, a near full-length structural model of the yeast  
387 Fzo1 was obtained using a totally integrative modeling approach (De Vecchis et al. 2017) (Fig. 1c).  
388 The model was experimentally validated. Although the rather low identity with BDLP, the study  
389 supported a comparable domain organization of the mitofusin Fzo1. Moreover, taking advantage of  
390 the yeast system, it was possible to validate the model through *in vivo* genetic assays using double  
391 swap mutations across predicted salt bridges that have been further corroborated by the human  
392 Mfn1 crystal structure (Cao et al. 2017; De Vecchis et al. 2017). In retrospect, this work depicts to  
393 date the most complete and elaborate model of mitofusins comprising both HB1 and HB2 helical  
394 bundles. In this system, the Fzo1 architecture is revealed with a precise description of the helical  
395 spans. In particular, HR1 and HR2 are not continuous but are locally bent over the hinges 1a, 1b.  
396 Moreover, the HR2 portion located in the trunk of Fzo1 is exposed to the solvent and may be  
397 available for *trans* tethering interactions (De Vecchis et al. 2017). In this context, the HR2 could  
398 have a role in membrane tethering as previously suggested for Mfn1 (Koshihara et al. 2004).  
399 However, alternative models are still possible for the human mitofusins, particularly after the recent  
400 new topology proposed, in which the HR2 would be exposed to the mitochondrial IMS (Mattié et  
401 al. 2018). Nevertheless, experimental evidence on Fzo1 suggested that HR1 and HR2 should run in  
402 parallel and are associated (De Vecchis et al. 2017). As opposed to the models presented above, the  
403 Fzo1 structural model was further characterized, experimentally validated and dynamically assessed  
404 in a membrane environment using a MD simulations approach (De Vecchis et al. 2017). The  
405 bundles HB1 and HB2 are linked by flexible hinges as in BDLP (Low and Löwe 2006; Low et al.  
406 2009). The study also clarifies the contribution of GTPase domain residues involved in nucleotide  
407 stabilization in line with previous annotations (Griffin and Chan 2006; Anton et al. 2011; Cao et al.  
408 2017). Overall, the Fzo1 architecture was somewhat anticipated since the initially hypothesized  
409 GTPase domain-dependent rearrangements (Cohen et al. 2011). The novel advantage of the Fzo1  
410 model is represented by the precise location of the TM domain. This novelty, in conjunction with  
411 the known GTPase-interface in mitofusins, allows one to correctly orient the Fzo1 model with  
412 respect to two juxtaposing bilayers to mime mitochondrial tethering. Due to partial structural data this  
413 is not yet possible for the human mitofusins (Qi et al. 2016; Cao et al. 2017). In light of that, we  
414 therefore started to investigate putative “open” and “closed” conformations based for instance, on  
415 the rearrangements proposed for the bacterial DLPs and guided by available distances between  
416 tethered mitochondrial membranes in human and yeast (Koshihara et al. 2004; Brandt et al. 2016).

417  
418 **A coarse-grained approach to study membrane-inserted mitofusin oligomers**

419

420 An essential requisite prior membrane fusion is the close apposition of two membrane bilayers, a  
421 mechanism known as tethering. Mitofusins are specialized in tethering opposing bilayers and  
422 promoting their fusion (Koshiba et al. 2004; Ishihara et al. 2004; Griffin and Chan 2006; Anton et  
423 al. 2011; Shutt et al. 2012; Cao et al. 2017). Similarly to other DRPs, mitofusins can self-assemble  
424 (Rapaport et al. 1998) and they do not function as monomers but oligomerize in both *cis* (on the  
425 same lipid bilayer) and *trans* (from opposing lipid bilayers) to mediate membrane attachment and  
426 fusion. In this respect, GTPase domain integrity is critical for the yeast mitofusin Fzo1 to determine  
427 *cis* and *trans* association (Hermann et al. 1998). When solubilized yeast mitochondria were  
428 subjected to size-exclusion chromatography, the mitofusin Fzo1 eluted in fractions that have been  
429 proposed to be compatible with a homodimer in *cis* (Anton et al. 2011). Further analyses have  
430 shown that Fzo1 dimerizes in an Ugo1-dependent manner and that the endogenous Fzo1 complex is  
431 homooligomeric (Anton et al. 2011). Crosslinking experiments have shown that purified Fzo1 could  
432 be crosslinked to an Fzo1 dimer (Anton et al. 2011). However, only the monomeric form is detected  
433 when mutants affect the GTP-binding site (Anton et al. 2011), leading to the conclusion that Fzo1  
434 dimerization relies on GTP binding but does not require GTP hydrolysis (Anton et al. 2011). Fzo1  
435 homodimers further associate upon formation of mitochondrial contacts and the corresponding  
436 oligomeric state has been proposed to be a *trans* tetramer (Rapaport et al. 1998; Anton et al. 2011).  
437 In particular, Fzo1 was shown to trigger the formation of a complex ring-shaped macromolecular  
438 complex (called MCD) during mitochondrial docking (Brandt et al. 2016).

439 Although the GTPase domain has often been the principal subject of attention, HR domains have  
440 been hypothesized to play an important role during mitochondrial tethering (Koshiba et al. 2004;  
441 Griffin and Chan 2006). As discussed previously, the HR2 helical segment was suggested as  
442 determinant for tethering (Koshiba et al. 2004; Franco et al. 2016). Interestingly, in our recent Fzo1  
443 model, the HR2 hydrophobic spine is largely exposed to the solvent, suggesting it could be  
444 available for putative interactions (De Vecchis et al. 2017). What still remains an open question is  
445 whether HRs or GTPase interactions take place in *trans* or in *cis* prior to mitochondrial tethering  
446 (Koshiba et al. 2004; Franco et al. 2016).

447

448 As introduced above we can take advantage of the current knowledge and the essential building  
449 blocks provided by our recent Fzo1 model (De Vecchis et al. 2017) to investigate *cis* and *trans*  
450 mitofusin oligomers. The models proposed in our study were further assessed for stability in a  
451 membrane environment using a coarse-grained MD simulation. In the context of mitofusins, these  
452 building blocks would form on the same membrane imposing the initial assembly of *cis*-oligomers.  
453 Consequently, we started our investigation from *cis*-assembled Fzo1 models that further gave rise to

454 3 *trans*-oligomers. We carefully consider current opinions on which domain should trigger the  
455 oligomerization in *cis*. Analogously to BDLP the GTPase of Fzo1 should have a predominant role  
456 (Low et al. 2009), though the same GTPase interface is thought to trigger *trans*-oligomerization of  
457 mitofusins (Cao et al. 2017; Yan et al. 2018). To take into account all possibilities, we also  
458 considered the HRs as possible actors to guide *cis*-oligomers in a parallel fashion although for Mfn1  
459 they have been observed to assemble in anti-parallel fashion in *trans* (Koshiba et al. 2004). The *cis*-  
460 models described above were combined in *trans*-oligomers and the model that particularly fits with  
461 the current literature is shown in Fig. 2a.

462

463 **Fig. 2** Putative Fzo1 *trans*-tethering complex. (a) The system represents the final configurations of  
464 the putative Fzo1 *trans*-tethering complex after a CG-MD simulation. The *cis* interactions occur  
465 towards the GTPase domain. The *trans* interactions occur through the HRs (also called HBs)  
466 oriented in an antiparallel fashion as suggested for the human Mfn1 (Koshiba et al., 2004).

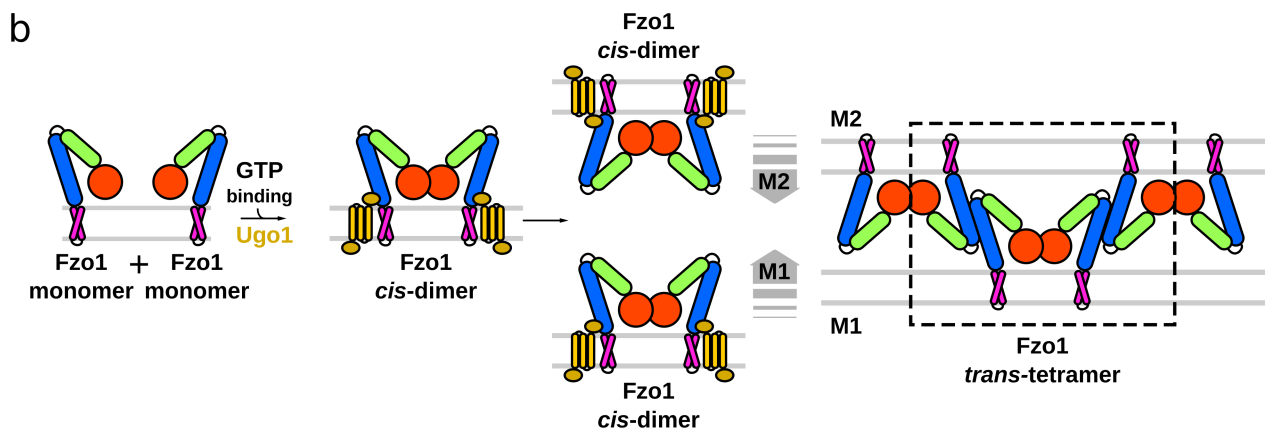
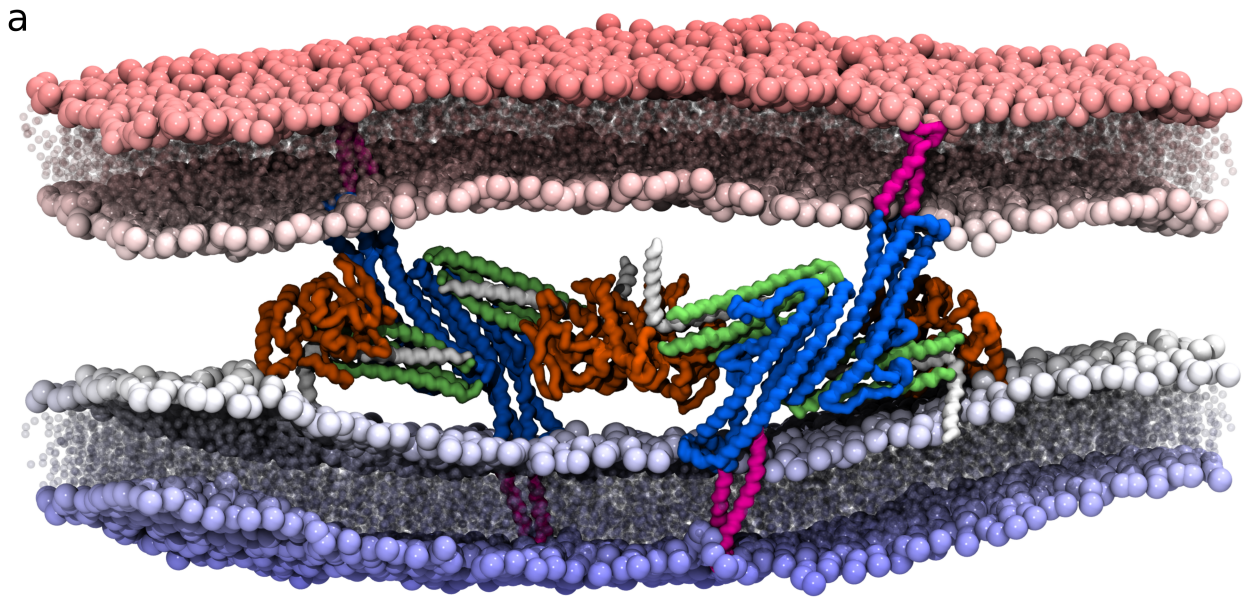
467 The color code is the same as in Fig. 1. GTPase domain, *orange*; HB1, *blue*; HB2 (or BSE), *green*;  
468 TM region, *pink*. The two bilayers represent two mitochondrial OM prior fusion (M1 and M2 in b).  
469 Lipid head groups and tails are shown as spheres from *red* to *blue* according the *z*-axis. Solvent is  
470 omitted for clarity. (b) Proposed Fzo1 fusion path. The color code is the same as in a

471

472 In this model two molecules of Fzo1 would oligomerize in *cis* through their GTPase domain (Fig.  
473 2b). The HB2/*trunk* region in the *cis*-oligomer would expose a hydrophobic spine from the HR2  
474 that would be available for *trans* interactions (De Vecchis et al. 2017). Consequently, the HRs  
475 spines will then be oriented in an anti-parallel fashion which is in agreement with the crystal  
476 structure of Mfn1 (Koshiba et al. 2004). The TM helices from each monomer also remained in  
477 contact with each other ( $88 \pm 4\%$  conservation) as well as stably embedded in the membrane during  
478 the simulation. The contact conservation respect to the lipid-TM contacts present in the initial setup  
479 was  $\sim 98 \pm 1\%$ . On top of this, the total lipid-protein contact surface increased about 25% during the  
480 simulation as a consequence of the observed membrane curvature.

481 Moreover, the tethering distance of 5.5 to 9.5 nm calculated during the MD simulations and  
482 imposed by this Fzo1 *trans*-oligomer would agree with the  $\sim 8$  nm separation between OMs  
483 observed experimentally (Brandt et al. 2016). It is finally possible that this Fzo1 *trans*-oligomer we  
484 proposed, might correspond to the macromolecular assembly observed in the ring-shaped  
485 mitochondrial docking complex (i.e. the MDC) were the tether distance reaches 6 to 8 nm (Brandt  
486 et al. 2016).

487





488 Our previous atomistic MD simulations of Fzo1 structural models have been conducted in a lipid  
489 bilayer which contains an equal concentration of phosphatidylethanolamine (POPE) and  
490 phosphatidylcholine (POPC). Despite the simplicity of this bilayer respect the native one in  
491 mitochondria (Zinser et al. 1991), we observed Fzo1 interacting more with POPE compared to  
492 POPC (De Vecchis et al. 2017). POPE plays an important role in fusion (Birner et al. 2001; Jahn  
493 and Grubmüller 2002; Joshi et al. 2012; Chan and McQuibban 2012) and is a major component of  
494 mitochondrial membranes, indeed PE-deficient yeast mitochondria that do fuse are notable for  
495 incompletely mixing bilayers (Chan and McQuibban 2012). An overlapping function in  
496 mitochondrial dynamics has been proposed for CL, which is specifically synthesized in  
497 mitochondria (Osman et al. 2009), where it plays a role in different mitochondrial processes  
498 (Pfeiffer et al. 2003; Osman et al. 2009). However, CL is also present in the OM (Zinser et al. 1991)  
499 where it has been hypothesized to play a role in mitochondrial OM dynamics (DeVay et al. 2009;  
500 Ban et al. 2010; Joshi et al. 2012; Zhang et al. 2014). The lipid-modifying enzyme belonging to the  
501 phospholipase D superfamily MitoPLD, targets the OM and promotes membrane adherence by  
502 hydrolyzing CL to generate phosphatidic acid (PA) (Choi et al. 2006; Jensen and Sesaki 2006; Roth  
503 2008; Kameoka et al. 2018), a common fusogenic lipid also required for SNARE-mediated fusion  
504 (Mendonsa and Engebrecht 2009). PA accumulation enhances mitofusin-dependent OM fusion  
505 (Choi et al. 2006). Such changes in lipid composition induce negative membrane curvature, and CL  
506 comprises about 20% of the total lipid content in mitochondrial IM, therefore it has been proposed  
507 to have a role in facilitating fusion and fission (Jensen and Sesaki 2006; Roth 2008; Joshi et al.  
508 2012; Ban et al. 2017). Although CL content in the OM is much lower than in the IM, it still  
509 represents 6% of the total lipids there (DeVay et al. 2009) and can approach 25% of the total lipid  
510 mixture in contact sites (Choi et al. 2006), where fusion may take place (Fritz et al. 2001).  
511 Therefore, it will be informative and auspicious in further MD simulation studies, to consider the  
512 effect of mitochondria lipidome as an emerging feature in membrane dynamics regulation.

513

## 514 **Conclusion**

515

516 Deciphering mitofusin's core functional assembly is a necessary prerequisite for an accurate  
517 understanding of the mitochondrial OM fusion pathway. In this review we highlight the importance  
518 of integrative modeling as a great advantage in the study of mitochondrial dynamics. The inherent  
519 domain modularity across the dynamin superfamily from bacterial dynamin to human homologues  
520 jointly with available biochemical data are actual resources. Their combination could reveal the  
521 essential building blocks of the different actors involved in mitochondrial dynamics. Further



522 combination of these information with the powerful tools of state-of-the-art computational  
523 methodologies, provides new possibilities and could contribute to advance the field of membrane  
524 dynamics.

525

## 526 **Compliance with Ethical Standards**

527

## 528 **Disclosure of potential conflicts of interest**

529 The authors declare no conflict of interest.

530

## 531 **Research involving Human Participants and/or Animals**

532 This research does not involve human participants or animals.

533

## 534 **References**

535

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