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Title:

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

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Abstract:

Mitochondria are dynamic organelles characterized by an ultrastructural organization which is essential in maintaining their quality control and ensure functional efficiency. The complex mitochondrial network is the result of the two ongoing forces of fusion and fission of inner and outer membranes. Understanding the functional details of mitochondrial dynamics is physiologically relevant as perturbations of this delicate equilibrium have critical consequences and involved in several neurological disorders. Molecular actors involved in this process are large

GTPases from the dynamin-related protein family. They catalyze nucleotide-dependent membrane remodeling and are widely conserved from bacteria to higher eukaryotes. Although structural characterization of different family members has contributed to understand molecular mechanisms of mitochondrial dynamics in more detail, the complete structure of some members as well as the precise assembly of functional oligomers remain largely unknown. As increasing structural data becomes available, the domain modularity across the dynamin superfamily emerged as a foundation for transfer the knowledge towards less characterized members. In this review we will first provide an overview of the main actors involved in mitochondrial dynamics. We then discuss recent example of computational methodologies for the study of mitofusin oligomers, and present how the usage of integrative modeling in conjunction with biochemical data can be an asset in progress the still challenging field of membrane dynamics.

Keywords:
Mitofusin, Fzo1, mitochondrial dynamics, mitochondrial fusion, mitochondrial fission, dynamin-related proteins.

Introduction

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

The main factors mediating mitochondrial dynamics are multi-domain mechano-chemical GTPases that belong to the dynamin superfamily (Praefcke and McMahon 2004; Ferguson and De Camilli 2012; Daumke and Praefcke 2016). These dynamin-related proteins (DRPs) are mostly implicated in membrane-remodeling processes (Daumke and Praefcke 2016; Ramachandran 2018).

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

OPA1(optic atrophy 1) and its yeast homologue Mgm1 (mitochondrial genome maintenance 1) mediate the fusion of the mitochondrial inner membrane (IM) and participate in cristae organization (Wong et al. 2000; Frezza et al. 2006). The fusion of the mitochondrial OM is triggered by the mitofusins Mfn1 and Mfn2 in mammals and by the fuzzy onion Fzo1 protein in yeast (Hales and Fuller 1997; Cohen and Tareste 2018). Notably, the phylogenetically related group of atlastin (and its yeast homologue Sey1p), functions in fusion of ER tubules (Hu et al. 2009; Orso et al. 2009). Several structural observations on recombinant atlastins and Sey1 have provided deep insights into fusion of ER tubules (Yan et al. 2015; Kim et al. 2017; Lee et al. 2019). In contrast, full-length mitofusins have not been purified which has limited the study of their assembly and oligomerization, which is an essential step in the OM fusion process (Rapaport et al. 1998; Fritz et al. 2001; Ishihara et al. 2003, 2004; Santel et al. 2003; Koshiba et al. 2004; Brandt et al. 2016)

Current developments in molecular modeling has made it possible to model and study homologous proteins whose structural details have not been elucidated experimentally. Moreover, advances in molecular dynamics simulations allow to study membrane proteins in a native-like lipid environment and overcome current limitations associated with their isolation and purification. Clear examples are widely discussed in recent review works (Friedman et al. 2018; Marrink et al. 2019; Corradi et al. 2019). The involvement of mitofusins in mitochondrial fusion was also recently reviewed (Cohen and Tareste 2018). Here, we thus review and discuss recent observations on mitofusin oligomerization and dynamics in their native membrane environment based on computational approaches such as molecular modeling and molecular dynamics (MD) simulation.

Mitochondrial fusion machineries

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

The second member of the DRP family required for mitochondrial IM fusion is Opa1/Mgm1 (Cipolat et al. 2004; Meeusen et al. 2006; Griparic et al. 2007; Kanazawa et al. 2008). They localize in the mitochondrial inter-membrane space (IMS) and are targeted to the IM thanks to an N-terminal mitochondrial import sequence (MIS) and transmembrane (TM) helices before the GTPase

domain (Wong et al. 2000; Olichon et al. 2002; Guillou et al. 2005). OPA1/Mgm1 are synthesized as precursors that undergo proteolytic processing (Herlan et al. 2003; Leroy et al. 2010). Two membrane-bound proteases, Oma1 (Ehses et al. 2009; Head et al. 2009) and Yme1l (Griparic et al. 2007; Song et al. 2007), process Opa1 resulting in fragments with different molecular weights and referred as "long" (L-OPA1) and "short" (S-OPA1). An initial study suggested that the two forms alone have little fusion activity and are both required for mitochondrial fusion (Song et al. 2007). However, it is now known that L-OPA1 is sufficient to deliver fusion (Tondera et al. 2009; Anand et al. 2014; Ban et al. 2017) and that there is a precise balance between the activity of both proteases Oma1 and Yme1l, though the exact role of S-OPA1 is not completely understood (MacVicar and Langer 2016). Recently a study suggested that S-OPA1 facilitates the binding of L-OPA1 to the negative charged lipid cardiolipin (CL), promoting fusion (Ban et al. 2017). This model also corroborates studies performed in yeast suggesting the recruitment of both L-OPA1 and S-OPA1 isoforms as well as CL for IM fusion (DeVay et al. 2009; Rujiviphat et al. 2009). Interestingly, OPA1-dependent fusion depends on Mfn1 but not Mfn2 (Cipolat et al. 2004; Tondera et al. 2009). Besides, a specific region "Mitofusin isoform-specific region" that confers mitofusin function has been identified (Sloat et al. 2019). This region provides a specific fusion activity to Mfn1 and Mfn2 and may be involved in higher order oligomerization. A functional connection between factors dedicated to OM and IM fusion is not surprising since the processes are coordinated. This observation is possibly corroborated by a recent new topology proposed for the human mitofusins according to which the C-terminal end is exposed to the mitochondrial IMS (Mattie et al. 2018). Nonetheless, other proteins, such as the yeast factor Ugo1, have been identified to have a possible role at the interface between the OM and IM.

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

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

Membrane fusion catalysts from bacterial homologues

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

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

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

In BDLP, as described for other dynamins (Ghosh et al. 2006; Chappie et al. 2010), the GTPase domain homodimerizes across the nucleotide binding pockets upon nucleotide binding (Low and Löwe 2006), resulting in the often called GG dimer (Chappie et al. 2010). However, the effect of nucleotide binding is not limited to GTPase domain homodimerization and GTP binding also induces lateral self-association between BDLP homodimers (Low et al. 2009). Trans oligomers have been proposed recently also for bacterial DLPs. Two chains, DLP1 and DLP2 from *Campylobacter jejuni,* form a *trans* tetramer through their helical bundles suggesting a mechanism that facilitates the tethering of membranes as suggested for Mfn1 (Liu et al. 2018).

Recent crystal structures of an Mfn1 fragment suggests a closed and open conformation (Qi et al. 2016; Cao et al. 2017; Yan et al. 2018). The protein construct consists of the GTPase domain linked to a 4-helix bundle which is comparable to the bacterial DLPs' *neck*. Mfn1 was found to dimerize in presence of GTP or with transition state analogues (i.e. GDP/BeF_3^- or GDP/AlF_4^-). Crystals were obtained with the transition state analogues and showed two alternative configurations as for BDLP, with the canonical GG interface followed by the 4-helix bundle (called HB1) (Cao et al. 2017; Yan et al. 2018). Interestingly, one alternative is reminiscent of the BDLP open form in which the HB1

bundle runs perpendicular to the GG interface (PDB: 5YEW (Yan et al. 2018)). Conversely, in the second alternative the HB1 runs parallel to the GG interface (PDB: 5GOM (Cao et al. 2017)) which is comparable to the BDLP closed form (Fig. 1c).

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

Topology and domain composition of mitofusins

Similar to other members of the dynamin superfamily, mitofusins have a modular domain organization confirmed also by a recent evolutionary analysis (Purkanti and Thattai 2015; Sinha and Manoj 2019) (Fig. 1a). They likely contain three distinct structural domains as described for the bacterial DLPs (Low and Löwe 2006): i) A GTPase domain (or G-domain) characterized by low nucleotide affinity, that binds and hydrolyzes GTP without guanine nucleotide exchange factor at variance with most G-proteins (Dever et al. 1987; Vetter and Wittinghofer 2001; Ozturk and Kinzy 2008) and two helical bundles probably involved in oligomerization and stimulation of GTPase activity ii) the HB1, or bundle signaling element (BSE) in human dynamin, or *neck* in bacterial dynamins (Low and Löwe 2006) and iii) the HB2, or *stalk* in eukaryotes, or *trunk* in bacterial dynamins (Fig. 1a). The GTPase domain mediates dimerization (Low and Löwe 2006; Low et al. 2009; Chappie et al. 2010; Anton et al. 2011; Cao et al. 2017; Yan et al. 2018) and harbors four conserved motifs called G1 to G4, involved in nucleotide binding with distinct characteristics. Remarkably, the G1 motif (also called P-loop), tightly wraps around the β-phosphate (Saraste et al. 1990; Chappie et al. 2010; Cao et al. 2017; Yan et al. 2018) and the G2, G3 motifs (also called the switch regions) change conformation upon GTP hydrolysis in human dynamin (Chappie et al. 2010). It has been hypothesized that the BSE domain bears possible functions in transferring the conformational information to the *trunk* region (Chappie et al. 2010), though the *stalk* domain is linked via a hinge to the GTPase domain in BDLP (Low et al. 2009). Additionally, many dynamins contain a domain for interactions with lipids. This can be a pleckstrin-homology (PH) domain as in the classical dynamins (Klein et al. 1998), a TM domain as in mitofusin/Fzo1 (Fritz et al. 2001; Rojo et al. 2002; De Vecchis et al. 2017; Mattie et al. 2018) and atlastin (Hu et al. 2009; Orso et al. 2009; Huang et al. 2017; Betancourt-Solis et al. 2018) or a *paddle* region for transient lipid attachment as in BDLP (Low et al. 2009) (Fig. 1a). This domain modularity across species is remarkable as taken singularly each protein may deliver completely different tasks that span from fusion to fission.

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

Mitofusin family members also contain two heptad repeat (HR) regions, called HR1 and HR2, situated as in Fzo1 on either side of the TM region. HR2 is proposed to be exposed to the mitochondrial IMS for the vertebrate mitofusins (Mattie et al. 2018). The yeast Fzo1 encodes a third HR (named HRN) located at the N-terminal end with respect to the GTPase domain (De Vecchis et al. 2017). The hydrophobic HR motifs are predicted to form coiled-coil structures (Eckert and Kim 2001) and are thought to play a critical role in tethering two mitochondria during a docking step before fusion (Koshiba et al. 2004; Daste et al. 2018). Such HRs are known to be central for other fusogenic machineries like viral fusion proteins (Skehel and Wiley 1998) and SNAREs (e.g. soluble N-ethylmaleimide-sensitive factor attachment protein receptor) (Ungar and Hughson 2003; Bonifacino and Glick 2004). However, the mechanism by which the human mitofusin HR1 promotes bilayer fusion appears to be different compared to SNAREs or viral proteins (Daste et al. 2018). In particular, the human HR1 possibly act by destabilizing the lipid bilayer especially in regions characterized by lipid packing defects (Daste et al. 2018). Structural data revealed that the HR2 region from Mfn1 (residues 660-735), (PDB : 1T3J (Koshiba et al. 2004)) forms a dimeric antiparallel coiled-coil that is 9.5 nm long (Koshiba et al. 2004). Its formation was hypothesized to be an important step during a *trans* complex mitofusin dimer assembly, as this structure would mediate tethering of mitochondria. Furthermore, the study found that removal of the GTPase domain (resulting in the construct comprising residues 331-741), promotes an extensive aggregation of mitochondria in a HR2-dependent manner (Koshiba et al. 2004). In comparison to this value of

9.5 nm observed by crystallography, electron microscopy revealed trapped densely packed mitochondria that maintain a uniform gap of ~16 nm between opposing OMs (Koshiba et al. 2004), whereas electron cryo-tomography of mitochondria isolated from *Saccharomyces cerevisiae* showed a ~8 nm separation between OMs (Brandt et al. 2016).

An integrated modeling approach for building intact mitofusin models

Several attempts have been made to model full-length structures of Fzo1 and human mitofusins because molecular modeling represents an alternative approach to investigate the possible architecture and dynamics of these large GTPases. Mitofusin closed and open conformations have been investigated using a mix of techniques from homology modeling to threading (Anton et al. 2013). In this study, full-length Fzo1 was modeled using the BDLP-apoenzyme as structural template (PDB: 2J68 (Low and Löwe 2006)), whereas a truncated variant without the TM domain was modeled using the open conformation of BDLP (PDB: 2W6D (Low et al. 2009)). In another study, the threading method was also employed to model the human Mfn1 using BDLP as a template (Escobar-Henriques and Anton 2013).

With similar approaches, a model of Mfn2 was subsequently proposed (Franco et al. 2016). In this model, HR1 and HR2 helical segments are locally unfolded and bent in the predicted hinge regions 1a and 1b. In this model the conformational change prior to tethering/fusion was proposed to consist in an unfolding process of the HR2 domain. This hypothesis clearly contrasts with the conformational switch seen for BDLP or for any other DRPs resolved so far (Low and Löwe 2006; Low et al. 2009; Michie et al. 2014; Liu et al. 2018) and has not been tested for thermodynamic stability. Therefore, this model made total abstraction of the established importance of the mitofusin GTPase domain in mitochondrial fusion (Low et al. 2009; Anton et al. 2011; Brandt et al. 2016). Nonetheless a short peptide (residues 367-384) designed to perturb intra-molecular HR1-HR2 interactions, was found to rescue mitochondrial morphology in murine embryonic fibroblasts and cultured neurons expressing either a Mfn2 mutant or the naturally occurring human Charcot-Marie-Tooth type 2A (CMT2A) mutant (Franco et al. 2016). It is unclear however whether the model presented in the study is the only one able to explain the experimental results. More recently a model of mitofusin 2 has been constructed using data from BDLP and Mfn1 and tested through a short molecular dynamics simulation (Beręsewicz et al. 2017, 2018).

In contrast to the mitofusin models mentioned above, a near full-length structural model of the yeast Fzo1 was obtained using a totally integrative modeling approach (De Vecchis et al. 2017) (Fig. 1c).

Despite the rather low identity with BDLP, the study supported a comparable domain organization of Fzo1. Moreover, taking advantage of the yeast system, it was possible to validate the model through *in vivo* genetic assays using double swap mutations across predicted salt bridges that have been further corroborated by the human Mfn1 crystal structure (Cao et al. 2017; De Vecchis et al. 2017). In retrospect, this work depicts to date the most complete and elaborate model of mitofusins comprising both HB1 and HB2 helical bundles. In this system, the Fzo1 architecture is revealed with a precise description of the helical spans. In particular, HR1 and HR2 are not continuous but are locally bent over the hinges 1a, 1b. Moreover, the HR2 portion located in the trunk of Fzo1 is exposed to the solvent and may be available for *trans* tethering interactions (De Vecchis et al. 2017). In this context, the HR2 could have a role in membrane tethering as previously suggested for Mfn1 (Koshiba et al. 2004). However, alternative models are still possible for the human mitofusins, particularly after the recent new topology proposed, in which the HR2 would be exposed to the mitochondrial IMS (Mattie et al. 2018). Nevertheless, experimental evidence on Fzo1 suggested that HR1 and HR2 should run in parallel and are associated (De Vecchis et al. 2017). As opposed to the models presented above, the Fzo1 structural model was further characterized, experimentally validated and dynamically assessed in a membrane environment using a MD simulations approach (De Vecchis et al. 2017). The bundles HB1 and HB2 are linked by flexible hinges as in BDLP (Low and Löwe 2006; Low et al. 2009). The study also clarifies the contribution of GTPase domain residues involved in nucleotide stabilization in line with previous annotations (Griffin and Chan 2006; Anton et al. 2011; Cao et al. 2017). Overall, the Fzo1 architecture was somewhat anticipated since the initially hypothesized GTPase domain-dependent rearrangements (Cohen et al. 2011). The novel advantage of the Fzo1 model is represented by the precise location of the TM domain. This novelty, in conjunction with the known GTPase-interface in mitofusins, allows one to correctly orient the Fzo1 model with respect to juxtaposing bilayers to mime mitochondrial tethering.

The importance of knowledge integration in the investigation of mitofusin oligomers

In this section, we will briefly present the current understanding and open questions about mitofusins/Fzo1 oligomers that were instrumental to and motivated the integrative modeling approach discussed in the next section.

The starting point is the observation that tethering is a crucial property of mitofusins/Fzo1. Indeed, mitofusins allow the tethering of opposing bilayers and promote their fusion (Koshiba et al. 2004;

Ishihara et al. 2004; Griffin and Chan 2006; Anton et al. 2011; Shutt et al. 2012; Cao et al. 2017). This phenomenon is an essential requisite prior to membrane fusion and to the close apposition of two membrane bilayers. In addition, similarly to other DRPs, mitofusins can self-assemble (Rapaport et al. 1998) and they do not function as monomers but oligomerize in both *cis* (on the same lipid bilayer) and *trans* (from opposing lipid bilayers) to mediate membrane attachment and fusion. Previous work suggests that Fzo1 can homodimerize in *cis* in a Ugo1-dependent manner (Anton et al., 2011). In this respect, GTPase domain integrity is critical for mitochondrial fusion (Hermann et al. 1998). Indeed, the importance of the GTPase domain in Fzo1 *cis* interactions, is shown by the observation that only the monomeric form is detected when there are mutations in the GTP-binding site (Anton et al., 2011). That is, Fzo1 dimerization relies on GTP binding although it does not require GTP hydrolysis (Anton et al. 2011). Therefore, the mitofusin Fzo1 *cis* complex, may be established before the hypothesized conformational switch possibly energized by the GTP. Finally, Fzo1 homodimers further associate upon formation of mitochondrial contacts and the corresponding oligomeric state has been proposed to be a *trans* tetramer (Rapaport et al. 1998; Anton et al. 2011). Current studies support the hypothesis that human mitofusins may establish *trans* interactions via the GTPase domain (Cao et al., 2017; Yan et al., 2018). However, the other critical determinant to mitofusin/Fzo1 function, the HRs, may be further involved in *trans*-interactions. HR domains have been hypothesized to play an important role during mitochondrial tethering (Koshiba et al. 2004; Griffin and Chan 2006). In particular, as discussed in the previous section, the human mitofusin HR2 helical segment was suggested as determinant for tethering (Koshiba et al. 2004; Franco et al. 2016). Interestingly, in the recent Fzo1 model, the HR2 hydrophobic spine is largely exposed to the solvent, suggesting it could be available for putative interactions also in the closed conformation (De Vecchis et al. 2017).

The distance between opposite mitochondrial membranes during tethering is also known from experiments. Fzo1 was shown to trigger the formation of a complex ring-shaped macromolecular complex during mitochondrial docking with an average inter-membrane distance of ~8 nm (Brandt et al. 2016). What still remains an open question is whether HRs or GTPase interactions take place in *trans* or in *cis* prior to mitochondrial tethering (Koshiba et al. 2004; Franco et al. 2016).

The systematic integration of the data presented above, allowed us to implement further modeling studies and start to investigate this question.

Molecular dynamics simulation approach to study membrane-inserted mitofusin oligomers

In the previous paragraph we highlighted the importance of knowledge integration as a route to organize the current understanding before applying modeling approaches. In this regard, a very recent study took advantage of the current knowledge and the essential building blocks provided by the Fzo1 model (De Vecchis et al. 2017) to investigate *cis* and *trans* mitofusin oligomers (Brandner, De Vecchis et al 2019). These building blocks were initially associated on the same membrane imposing the initial assembly of *cis*-oligomers. *Cis*-assembled Fzo1 models were further assembled to give rise to 3 *trans*-oligomers. The model that particularly fits with the current literature is shown in Fig. 2a.

The model was further simulated in a coarse-grained molecular dynamics (CG-MD) simulation to assess its stability and dynamics in a lipid environment. In this model two molecules of Fzo1 would oligomerize in *cis* through their GTPase domain (Fig. 2b). The HB2/*trunk* region in the *cis*-oligomer would expose a hydrophobic spine from the HR2 that would be available for *trans* interactions (De Vecchis et al. 2017). Consequently, the HRs spines will then be oriented in an anti-parallel fashion which is in agreement with the crystal structure of Mfn1 (Koshiba et al. 2004). During the CG simulation, the TM helices from each monomer also remained in contact with each other (88 ± 4% conservation) as well as stably embedded in the membrane during the simulation. The contact conservation respect to the lipid-TM contacts present in the initial setup was ~98 ± 1%. On top of this, the total lipid-protein contact surface increased about 25% during the simulation as a consequence of the observed membrane curvature.
Moreover, the tethering distance of 5.5 to 9.5 nm calculated during the MD simulations and imposed by this Fzo1 *trans*-oligomer would agree with the ~8 nm separation between OMs observed experimentally (Brandt et al. 2016). It is finally possible that this Fzo1 *trans*-oligomer we proposed, might correspond to the macromolecular assembly observed in the ring-shaped mitochondrial docking complex (MDC) where the tether distance reaches ~8 nm (Brandt et al. 2016).

Future perspectives

Determining the complete structure of the mitofusins/Fzo1 would represent a breakthrough in the study of mitochondrial fusion. Current difficulties are mainly represented by the protein purification and crystallization. However, one possible road to challenge these limitations is integrative homology modeling. Importantly, even though structural data may become available in the near future, a deep comprehension of mitofusin structure-function relationships, will necessarily involve

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

Conclusion

Deciphering mitofusin's core functional assembly is a necessary prerequisite for an accurate understanding of the mitochondrial OM fusion pathway. In this review we highlight the importance of integrative modeling as a great advantage in the study of mitochondrial dynamics. The inherent

domain modularity across the dynamin superfamily from bacterial dynamin to human homologues jointly with available biochemical data are actual resources. Their combination could reveal the essential building blocks of the different actors involved in mitochondrial dynamics. Further combination of these information with the powerful tools of state-of-the-art computational methodologies, provides new possibilities and could contribute to advance the field of membrane dynamics.

Compliance with Ethical Standards

Disclosure of potential conflicts of interest

The authors declare no conflict of interest.

Research involving Human Participants and/or Animals

This research does not involve human participants or animals.

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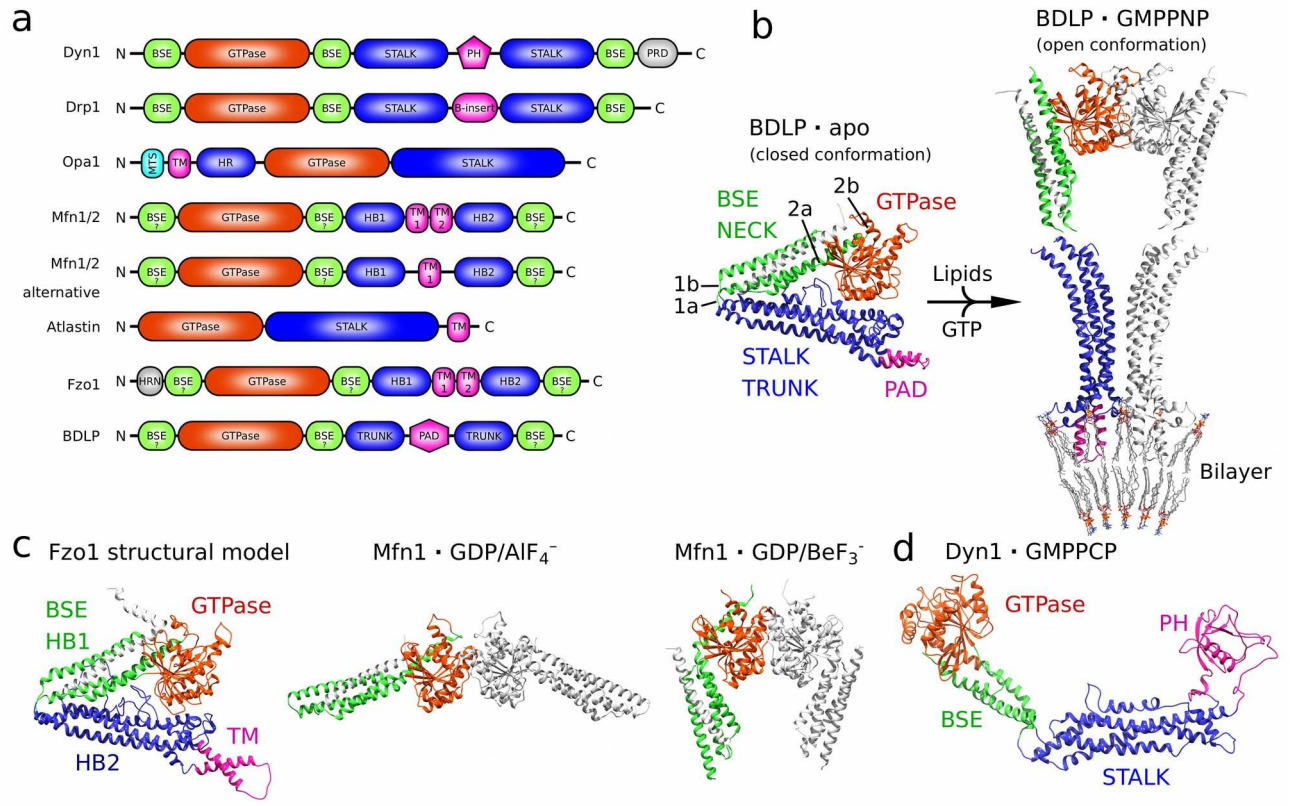

Fig. 1 Schematic representation of the modular domain architecture of selected proteins from the dynamin superfamily. (a) Cartoon summarizing the modular domain architecture for selected DRPs and DLPs discussed in the text. The classical nomenclature is indicated. The heptad repeats of the mitofusins/Fzo1 (HRN, HR1 and HR2) are also called HB1 and HB2 (Helical Bundles). They are aligned to the *stalk*/*neck* domains of other DLPs and dynamins showing a similar architecture and they might serve as BSE (Bundle Signaling Element). MTS (Mitochondrial Targeting Sequence), TM (transmembrane), PAD (paddle), PH (Pleckstrin Homology), PRD (Proline-Rich Domain). (b) The BDLP conformational change upon GTP and lipid binding. Hinges 1a, 1b, 2a and 2b are indicated. The structure is closed in its GDP-bound (not shown) and in its apo-form (left) (PDB: 2J68 (Low and Löwe 2006)). Lipid binding concomitant with GTP hydrolysis, reveals a conformational change detected using cryo-electron microscopy (right) (PDB: 2W6D (Low et al. 2009)). (c) (Left) The Fzo1 structural model (De Vecchis et al. 2017) with HB1 and HB2. (Center) Crystal structure of the human Mfn1 fragment-construct obtained upon addition of GDP/AlF_4^- (not shown) (PDB: 5GOM (Cao et al. 2017)) and observed in presence of GDP/BeF_3^- (not shown) (PDB: 5YEW (Yan et al. 2018)) (Right). (d) Cryo-electron microscopy of the human dynamin 1 (Dyn1) in complex with GMPPCP (not shown) (PDB: 6DLU (Kong et al. 2018)). The PRD was not present in the reconstruction

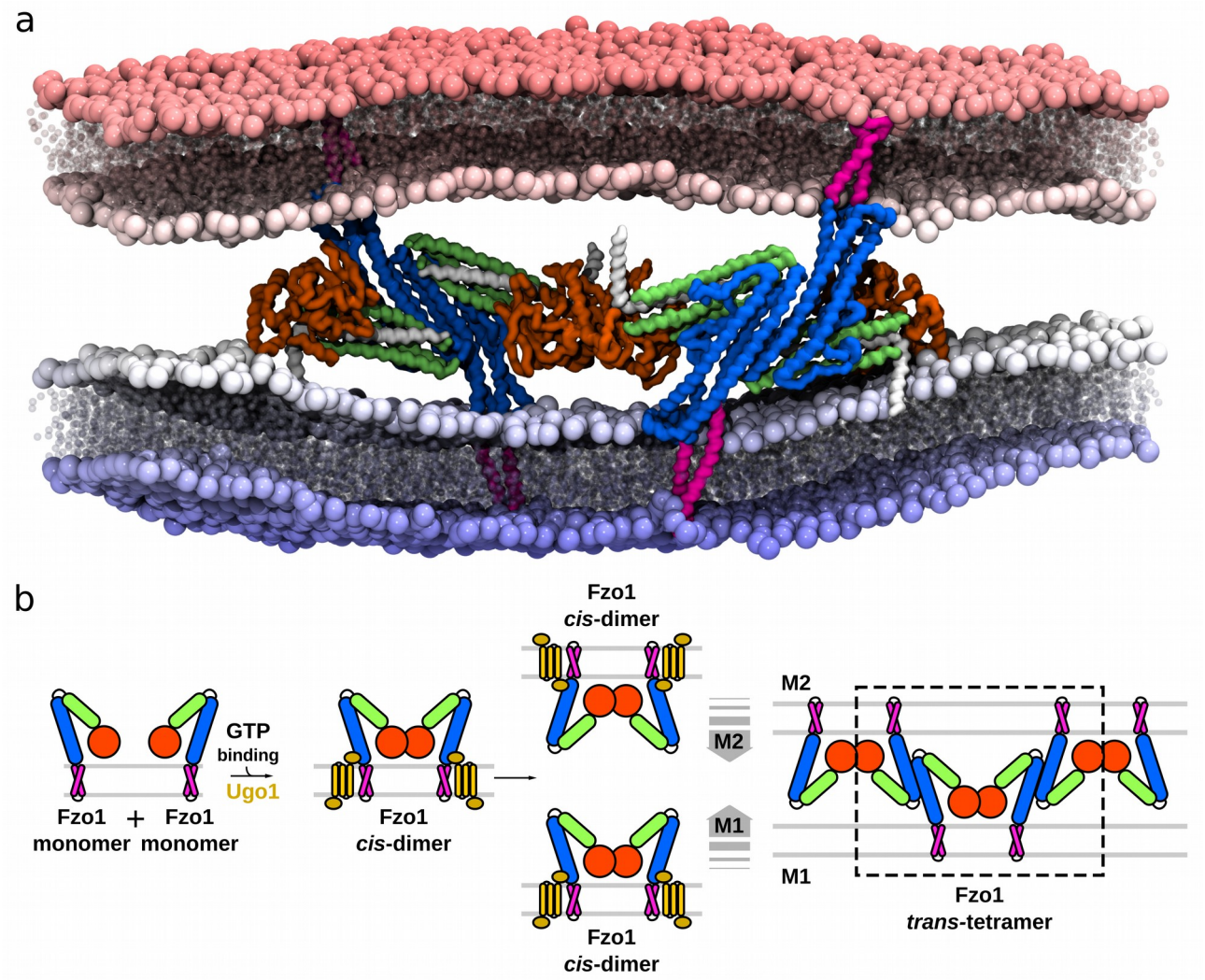

Fig. 2 Putative Fzo1 trans-tethering complex. (a) The system represents the final configurations of the putative Fzo1 *trans*-tethering complex after a CG-MD simulation. The *cis* interactions occur towards the GTPase domain. The *trans* interactions occur through the HRs (also called HBs) oriented in an antiparallel fashion as suggested for the human Mfn1 (Koshiba et al. 2004). The color code is the same as in Fig. 1. GTPase domain, *orange*; HB1, *blue*; HB2 (or BSE), *green*; TM region, *pink*. The two bilayers represent two mitochondrial OM before fusion (M1 and M2 in b). Lipid head groups and tails are shown as spheres from *red* to *blue* according the *z*-axis. Solvent is omitted for clarity. (b) Proposed Fzo1 fusion path. The color code is the same as in a