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Physics-based oligomeric models of the yeast mitofusin Fzo1 at the molecular scale in the context of membrane docking

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1 **Title**
2 **Physics-based oligomeric models of the yeast mitofusin Fzo1 at molecular-**
3 **scale in the context of membrane docking.**
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19 **ABSTRACT**

20

21 Tethering and homotypic fusion of mitochondrial outer membranes is mediated by large GTPases
22 of the Dynamin-Related Proteins family called the mitofusins. The yeast mitofusin Fzo1 forms high
23 molecular weight complexes and its assembly during membrane fusion likely involves the
24 formation of high order complexes. Consistent with this possibility, mitofusins form oligomers in
25 both *cis* (on the same lipid bilayer) and *trans* to mediate membrane attachment and fusion.

26 Here, we rely on our recent Fzo1 model to investigate and discuss the formation of *cis* and *trans*
27 mitofusin oligomers. We have built 3 *cis*-assembly Fzo1 models that gave rise to 3 distinct *trans*-
28 oligomeric models of mitofusin constructs. Each model involves two main components of mitofusin
29 oligomerization: the GTPase and the trunk domains. The oligomeric models proposed in this study
30 were further assessed for stability and dynamics in a membrane environment using a coarse-grained
31 molecular dynamics (MD) simulation approach. A narrow opening ‘head-to-head’ *cis*-
32 oligomerization (via the GTPase domain) followed by the antiparallel ‘back-to-back’ *trans*-
33 associations (via the trunk domain) appears to be in agreement with all the available experimental
34 data. More broadly, this study opens new possibilities to start exploring *cis* and *trans* conformations
35 for Fzo1 and mitofusins in general but also for other fusion-DRPs.

36 INTRODUCTION

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Mitochondria are dynamic organelles organized as a cytoplasmic reticulum. Mitochondria fuse their outer and inner membranes to form tubules. These mitochondrial tubules can interconnect through fusion but can also fragment through fission of their membranes to yield a network with remarkable plasticity. Together, fusion and fission thus regulate the whole morphology and dynamics of the mitochondrial network, which makes these processes essential for maintenance of mitochondrial integrity and consequently all mitochondrial functions (Friedman and Nunnari, 2014; Shutt and McBride, 2013; Tilokani et al., 2018; Westermann, 2010).

From a mechanistic point of view, mitochondrial fission as well as fusion of outer and inner membranes are all mediated by members of the Dynamin-Related Proteins family (Ramachandran, 2018). These large GTPases act as remodelers of intracellular lipid bilayers through two properties: their capacity to bind biological membranes and their propensity to oligomerize into high order macromolecular structures. Consistent with these features, fission DRPs are recruited to mitochondrial outer membranes by specific adaptor proteins, where they auto-oligomerize upon binding of GTP to assemble into macromolecular spirals that wrap around mitochondrial tubules (Tilokani et al., 2018). Subsequent GTP hydrolysis induces conformational rearrangements of the dynamins, which result in reduced diameter of the spirals and constriction of mitochondrial tubules, followed by their separation.

Fusion-DRPs, on the other hand, are transmembrane proteins that promote homotypic merging of the lipid bilayers in which they are inserted (Cohen and Tareste, 2018). The mitofusins Mfn1 and Mfn2 (Fzo1 in yeast) fuse mitochondrial outer membranes whereas fusion between inner membranes is mediated by Opa1 (Mgm1 in yeast). Another key member of fusion DRPs is the Atlastin ATL-1 (Sey1 in yeast) that merges membranes from ER tubules. Mitofusins and Atlastins have in common to auto-oligomerize in *trans* (from opposing lipid bilayers) and in a GTP binding and hydrolysis-dependent manner to tether opposing lipid bilayers and promote their fusion (Cohen and Tareste, 2018). Recent crystal structures of protein portions lacking the TM regions of ATL-1, Sey1 and MFN1 further allow depicting the assembly of DRPs during membrane fusion as protein dimers interacting in *trans* through their respective GTPase domains (Bian et al., 2011; Byrnes et al., 2013; Cao et al., 2017; Moss et al., 2011; Yan et al., 2018; Yan et al., 2015). However, the yeast mitofusin Fzo1 also forms high molecular weight complexes (Rapaport et al., 1998). In particular, Fzo1 was shown to trigger the formation of a ring-shaped macromolecular complex during mitochondrial docking (Brandt et al., 2016), suggesting that DRPs assembly during membrane fusion might involve the formation of high order complexes rather than solely *trans* homodimers.

Consistent with this possibility, mitofusins form oligomers in both *cis* (on the same lipid bilayer) and *trans* to mediate membrane attachment and fusion (Santel et al., 2003)(Ishihara et al., 2004; Griffin and Chan, 2006; Cao et al., 2017; Koshiba et al., 2004; Anton et al, 2011; Shutt et al., 2012). Besides the GTPase domain, mitofusins also include two heptad-repeat domains (HR1 and HR2) that may get involved in homotypic interactions during mitochondrial tethering (Koshiba et al., 2004; Griffin and Chan, 2006; De Vecchis et al., 2017). Whether GTPase and HR domain interactions take place in *cis* or *trans* before or during mitochondrial tethering remains to be investigated or confirmed (Franco et al., 2016; Koshiba et al., 2004).

Here, we rely on our recent Fzo1 model (De Vecchis et al., 2017) to investigate and discuss distinct hypotheses for the formation of *cis* and *trans* mitofusin oligomers. Besides being the only near full-length model as of today, it contains a membrane domain, which is a requisite for investigating mitofusin orientations in a bilayer. We employed a modelling procedure that has been guided by available experimental data from the literature. The models proposed in this study were further assessed for stability and dynamics in a membrane environment using a coarse-grained molecular dynamics (MD) simulation approach. This study opens new possibilities to start exploring *cis* and *trans* conformations for Fzo1 and mitofusins in general but also for other fusion-DRPs.

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METHODS

The modelling work presented here builds upon an experimentally validated model of the monomeric unit, namely our previously published model of Fzo1 in a closed conformation (De Vecchis et al., 2017). It is first used to generate a monomeric model of the open conformation. Those two models are the basis for the construction of Fzo1 dimer models in *cis* and then tetramers, via the dimerization in *trans* of Fzo1 *cis*-dimers.

Modelling the Fzo1 GTPase dimer construct

Two chains of the Fzo1 model in closed conformation (De Vecchis et al., 2017) were placed by superimposing their GTPase domains onto that of BDLP in open conformation (PDB-id 2W6D, Low et al., 2009). Then, only the coordinates of the two fragments that comprise the Fzo1 GTPase domain (res 188-461) were retained to form the final GTPase domain dimer model. The loop refinement tool implemented in MODELLER (Fiser et al., 2000) was used to remove a clash in both chains involving an unresolved loop in the template 2J68 (Low and Löwe, 2006) (res 215-219). Models were ranked according to the discrete optimized protein energy (DOPE) method (Shen and Sali, 2006), selecting the best-scoring loop out of 20 candidates.

Modelling *cis*-dimer configurations

The Fzo1 head-to-head *cis*-dimer. Two Fzo1 chains in closed conformation (De Vecchis et al., 2017) were oriented facing each other within a compatible distance to accommodate two interacting GTPase domains. Subsequently, the coordinates of residues 188-440 enclosed between hinges 2a, 2b (i.e. comprising the GTPase domain) were removed from both chains and replaced with the GTPase dimer construct described above. The latter was manually positioned between the two deleted chains resulting in the head-to-head interaction dimer (Fig. 2b). The backbone interruptions were connected using the loop refinement tool implemented in MODELLER (Fiser et al., 2000) using positions 185,188 and 436,445 as anchors. Solutions were ranked according to the DOPE method (Shen and Sali, 2006), selecting the best-scoring loop out of 20 candidates.

The Fzo1 back-to-back *cis*-dimer. Two chains of the Fzo1 model in closed conformation (De Vecchis et al., 2017) were manually oriented with respect to each other in order to generate the back-to-back interaction (discussed in the text). In the resulting model system (Fig. 2c) the HR domains face each other in a parallel fashion.

The Fzo1 open *cis*-dimer. The coordinates from BDLP in open conformation (PDB-id 2W6D, Low et al., 2009) derived from the electron density map of native BDLP lipid tubes (accession code: EMD-1589) were used as template to model Fzo1 in open conformation. Starting from our previous Fzo1-BDLP target-template alignment (De Vecchis et al., 2017) and using an analogous approach to the one described in Low et al., 2009, we introduced homologous chain breaks on the Fzo1 model (De Vecchis et al., 2017), resulting in five rigid blocks (Fig. 1a). Each fragment was superposed to its corresponding fragment in 2W6D, in order to reconstitute the orientation found in BDLP (Fig. 1). The MatchMaker tool from the UCSF Chimera software (Pettersen et al., 2004) was used during this procedure. The loop refinement tool implemented in MODELLER (Fiser et al., 2000) enabled us to complete the model in the resulting backbone interruptions and to remove a clash in both chains between the side chain of the Lys271 and the backbone of the Ala401 residues, using positions 268 and 273 as anchors. Solutions were ranked according to the discrete optimized protein energy (DOPE) method (Shen and Sali, 2006), selecting the best-scoring loop out of 10 models.

Modelling the *trans*-dimer configurations

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The Fzo1 head-to-head trans tethered tetramer in open conformation. Two Fzo1 open *cis*-dimer models obtained as described above were manually oriented to mimic the *interactions* in *trans* towards their respective GTPase domains. In the resulting model system the two transmembrane segments are located at opposite ends (Fig. 5a).

The Fzo1 head-to-head trans tethered tetramer (antiparallel). The transmembrane segment of two Fzo1 head-to-head *cis*-dimers described above were manually oriented at opposite ends in order to optimize the interaction between their respective HR domains oriented in an antiparallel fashion (Fig. 5c). Note that this system, although antiparallel, could also be considered as back-to-back.

The Fzo1 head-to-head trans tethered tetramer (parallel). Two Fzo1 back-to-back *cis*-dimers obtained as described above were initially positioned with the respective transmembrane segments at opposite ends in order to mimic the supposed tethering process. Subsequently, the coordinates of residues 101-491 and 816-855 enclosed between hinges 1a, 1b (i.e. comprising the GTPase domain and the 3-helix bundle), were removed from the two resulting juxtaposing chains. Then, in a similar way the GTPase dimer construct was built (see above), we superposed the GTPase domain alpha carbons of two Fzo1 chains in closed conformation (De Vecchis et al., 2017) with the human mitofusin dimer (PDB-Id: 5GOM, Cao et al., 2017). This choice was motivated and directly inspired by the work from Gao and collaborators that proposed a possible Mfn1 *trans* cross oligomer (Cao et al., 2017). From the resulting Fzo1 dimer, only the GTPase domain and the 3-helix bundle were selected and used to replace the aforementioned deleted portions, then generating the *trans* head-to-head interaction (Fig. 5b). A clash in one chain (res 215-218) was removed from the resulting Fzo1 dimer using the loop refinement tool implemented in MODELLER (Fiser et al., 2000). Models were ranked according to the discrete optimized protein energy (DOPE) method (Shen and Sali, 2006), selecting the best-scoring loop out of 10 candidates. The same tool was used to reconstitute the backbone interruptions. Positions 491,495 and 812,816 were selected as anchors. The best-scoring loop was selected out of 10 candidates.

Molecular Dynamics.

System setup and parameters.

171 Topologies to run coarse-grained (CG) simulations were generated with the martinize tool choosing
172 the Martini v.2.1 force field with elastic network (de Jong et al., 2013; Monticelli et al 2008). The
173 force bond constant was set to $500 \text{ kJ mol}^{-1} \text{ nm}^{-2}$ with lower and upper elastic bond cutoffs of 0.5
174 and 0.9 nm respectively. Firstly, 5000 steps of steepest descent with position restraints for the
175 protein were run followed by 5000 steps without restraints. The obtained coordinates were inserted
176 in a POPC:POPE (1:1) membrane via the insane tool (Wassenaar et al., 2015), where the membrane
177 position was manually set up to match the reported transmembrane regions corresponding to
178 residues 706-726 and 737-757 according to UniProt numbering (De Vecchis et al., 2017). All
179 systems were fully solvated to mimic an environment of 150 mM of NaCl solution. See
180 Supplementary Table 1 for more details about each simulation setup.

181 All the final systems followed the same simulation protocol using the GROMACS 5.0.4 software
182 (Abraham et al., 2015). Further 5000 steps of steepest descent minimisation with position restraints
183 of $1000 \text{ kJ mol}^{-1} \text{ nm}^{-2}$ in protein and lipids were followed by 5000 steps without position restraints.
184 Equilibration was performed in three stages, with timesteps of 20 fs. Firstly, 25000 steps of
185 equilibration were run at 310 K using the V-rescale thermostat (Bussi et al., 2007) and semi-
186 isotropic pressure coupling via Berendsen barostat (Berendsen et al., 1984) with position restraints
187 of $1000 \text{ kJ mol}^{-1} \text{ nm}^{-2}$ for protein and lipids, followed by the same setup without position restraints.
188 Finally, the last equilibration step was run for 50000 steps with the V-rescale thermostat (coupling
189 constant $\tau_t = 1 \text{ ps}$) and semi-isotropic coupling with Parinello-Rahman barostat (Parinello and

190 Rahman, 1981) (coupling constant $\tau_p = 12$ ps). Production runs were 1 μ s long for all the six
191 systems, following the same parameters as those used in the last equilibration setup.

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193 **Analysis.**

194 Root mean square deviations were calculated in GROMACS 5.0.4 via the gmx rms tool considering
195 only backbone beads. Interfaces were obtained based on differences between solvent accessible
196 surface areas obtained with the gmx sas tool, using a probe radius of 0.2638 nm in agreement with
197 the CG water size. An interface between e.g protein A and B ($I_{A/B}$) was calculated as the sum of the
198 surface areas of protein A and protein B in the interface, following the equation: $I_{A/B} = I_A + I_B =$
199 $(SASA_A - SASA_{A_complex}) + (SASA_B - SASA_{B_complex})$ where $SASA_A$ is the solvent accessible surface
200 area calculated considering the protein A as isolated and $SASA_{A_complex}$ is the solvent accessible
201 surface area calculated for the protein A considering its solvent accessibility in the complex.

202 The distance in the membrane-normal Z axis between the phosphate beads from both bilayers
203 (oriented in the x,y plane) was measured in blocks (grid in x,y of size ~ 20 Å). Z coordinates of
204 atoms corresponding to each block were averaged and subsequently, the distance between the
205 average values of each block from the upper bilayer was subtracted from the lower one. Resulting
206 difference inter-membrane distances between the final and initial structure of the production run are
207 shown as a matrix.

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210 **RESULTS AND DISCUSSION**

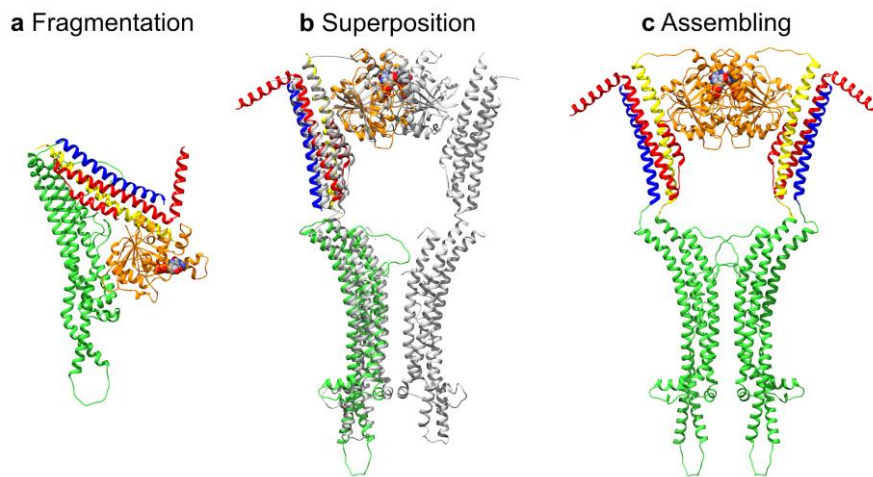
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212 **Putative models for Fzo1 cis-dimers**

213 Mitochondrial tethering requires oligomerization of mitofusins in *cis* and then in *trans* (Anton et al.,
214 2011; Fritz et al., 2001; Ishihara et al., 2004; Ishihara et al., 2003; Koshiba et al., 2004; Rapaport et
215 al., 1998; Santel et al., 2003). We thus reasoned that focusing on *cis*-oligomerization aspects in first
216 instance might facilitate investigating their general properties of assembly. In this regard, the
217 Bacterial Dynamin Like Protein from *Nostoc punctiforme* represents a starting point of choice.
218 BDLP was not only used as the template to generate our previous Fzo1 structural model (De
219 Vecchis et al., 2017) but this bacterial DRP also displays well established *cis*-oligomerization
220 properties (Low and Lowe, 2006; Low et al., 2009). Upon binding of non-hydrolysable analogues
221 of GTP, BDLP operates an extensive conformational change from a compact structure (that was
222 used as the template to generate the closed Fzo1 model, Fig. 1a) to an extended conformation. This
223 extended conformation allows insertion of BDLP into lipid bilayers through a membrane paddle
224 and favors its *cis*-oligomerization through GTPase domain interactions (PDB-id 2W6D, Low et al.,
225 2009). The Fzo1 open-conformation model was obtained by superimposing our previously
226 published closed conformation model piecewise onto the open structure of BDLP (Fig. 1b; see
227 Methods for details). The final model of the Fzo1 dimer in open conformation was then superposed
228 with respect to the template structure 2W6D using the C α atoms (Fig. 1c). The resulting structural
229 drift, measured as RMSD between target and template, is rather low at 0.63 Å, which underlines the
230 similarity of both molecular systems.

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Figure 1. Key steps of the modelling workflow to build a model of Fzo1 in open conformation, based on the BDLP structure. (a) The Fzo1 structural blocks delimited according to the putative hinges proposed for BDLP are highlighted by different colors. The blocks are coloured in rainbow from N-terminal (*red*) to C-terminal (*blue*). (b) Each block in Fzo1 (colored parts) was superposed to its corresponding fragment in 2W6D (*gray* ribbon). Note the backbone interruptions in the structure. (c) The final Fzo1 model in open conformation. Note that each GTPase domain (*orange*) from both chains is in close contact with each other. The GDP nucleotide is shown in space-filling representation.

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The BDLP-like *cis*-oligomerization model (Fig. 2a) imposes an extensive conformational switch of Fzo1 with an opening angle of about 180° between the four-helix bundle and the trunk of the mitofusin. While a possible conformational reorganization has been experimentally documented in this hinge region of Fzo1 (Cohen et al., 2011), a significantly lower angle of opening may not be excluded. GTPase domain contacts observed for human mitofusin (Cao et al., 2017) provide a template to form dimers with such a low opening angle (Fig. 2b). This assembly leads to a distinct *head-to-head* orientation with notably narrow Fzo1 opening that differs from the significantly wide Fzo1 opening seen in the BDLP-like *cis*-oligomerization model.

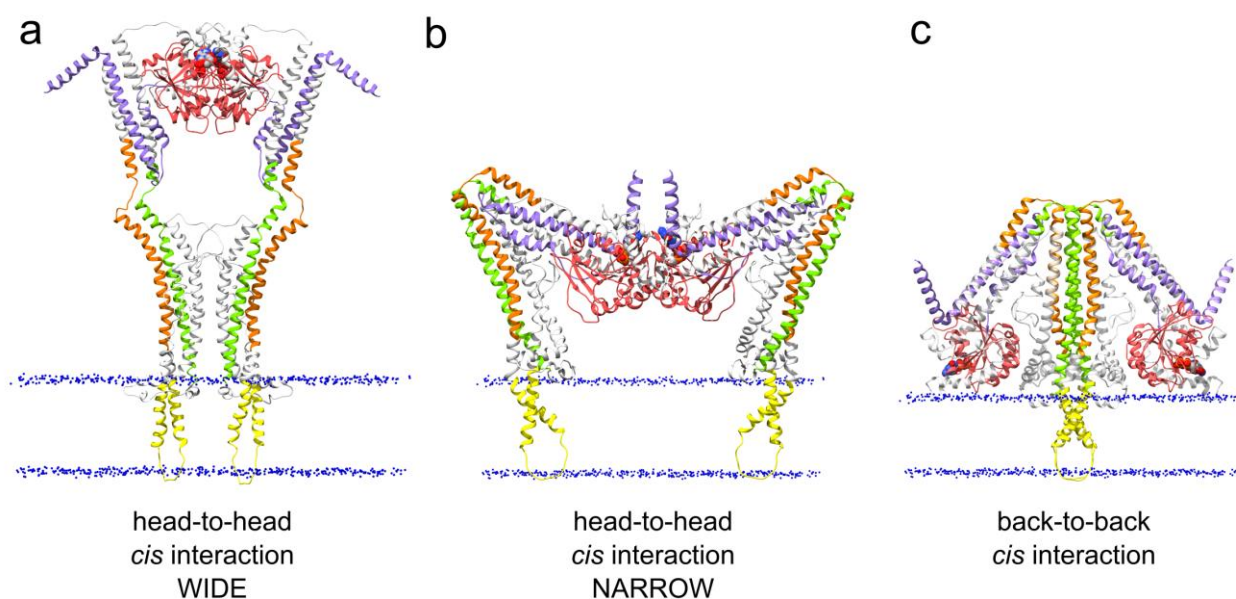
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In either the wide or narrow *head-to-head* models, the GTPase domain interface favours *cis*-dimerization of Fzo1 after GTP binding. This contrasts with the current view that oligomerization of mitofusins through their GTPase domain promotes their association in *trans* rather than *cis* (Cao et al., 2017; Yan et al., 2018). In this latter scenario, *cis*-oligomerization should thus employ Fzo1 interaction regions distinct from the GTPase domain. Interestingly, in our recent Fzo1 model, HR2 is exposed to the solvent suggesting it could be available for putative hydrophobic interactions with neighbouring Fzo1 molecules (De Vecchis et al., 2017). The coiled-coil structures would be positioned *back-to-back* to yield a *cis*-dimer with GTPase domains available for interactions in *trans* (Fig. 2c). This would notably be consistent with the crystal structure of the Mfn1 HR2 domain (Koshiba et al., 2004) that supports the possibility that mitofusins could also associate through their trunk region. However, in this crystal (PDB-id 1T3J), the interacting HR2 domains adopt an antiparallel-orientation suggesting that the transmembrane segments of two mitofusin molecules interacting through their trunks would be located on opposite membranes.

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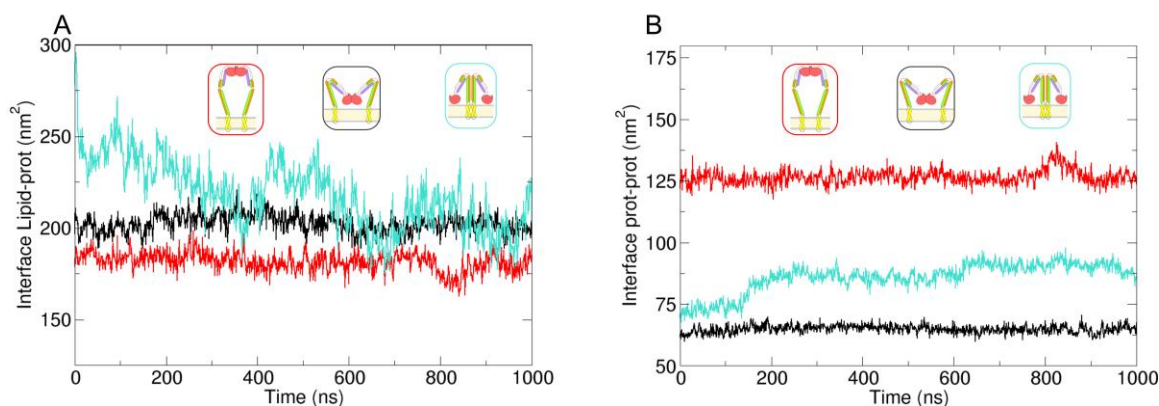
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Figure 2. Putative Fzo1 models for interaction in *cis* configuration. **a** and **b.** Wide and narrow head-to-head complexes, respectively, in which two Fzo1 molecules interact via their GTPase domains, consistent with interactions observed for the bacterial BDLP (Low and Löwe, 2006; Low et al., 2009) and human mitofusin (Cao et al., 2017). **c.** back-to-back complex for the closed model where two Fzo1 molecules interact through their HR domains. The domains highlighted by color are: *violet*, HRN; *green*, HR1; *orange*, HR2; *red*, GTPase and *yellow*, transmembrane. Phosphorus atoms (*blue*) from lipid bilayer headgroups and GDP nucleotide are depicted in the space-filling representation.

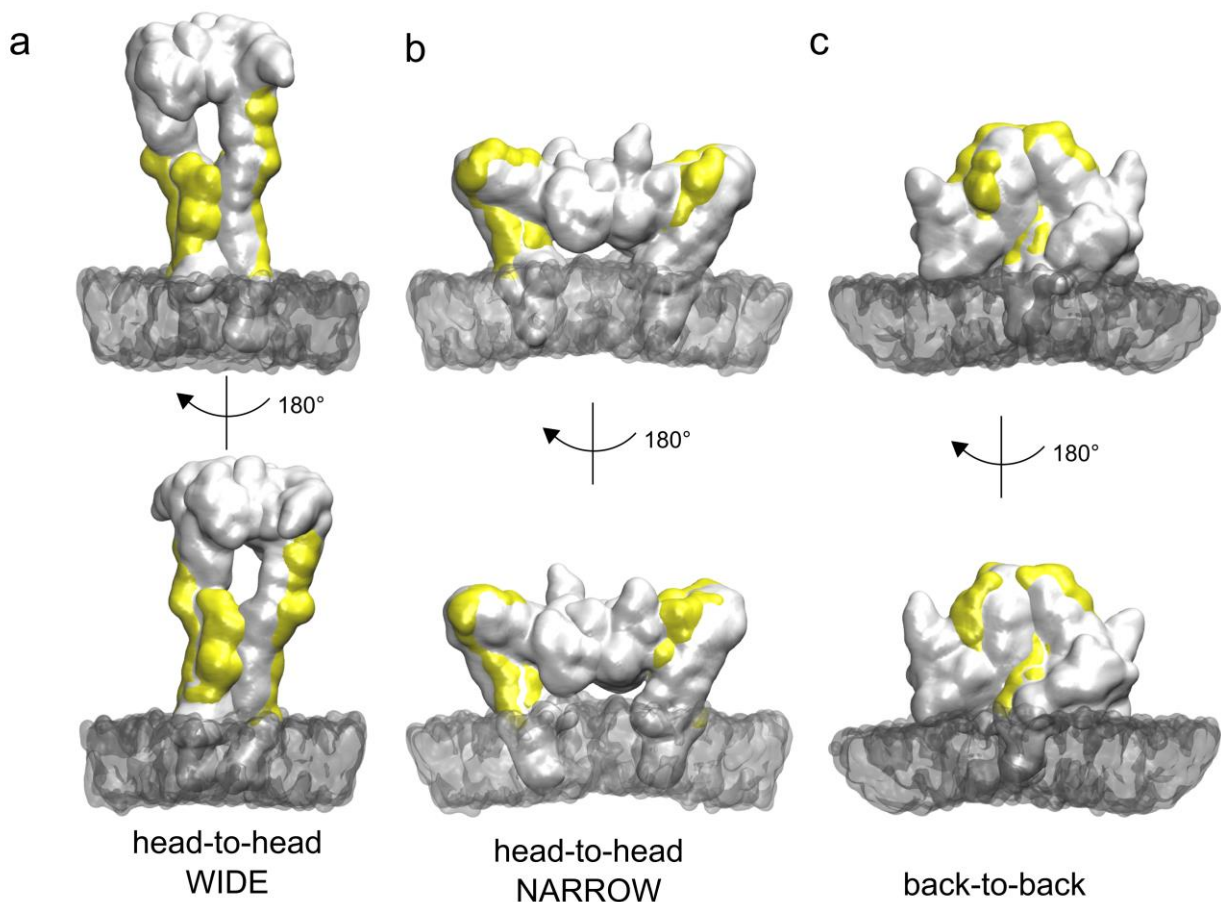


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Figure 3. Lipid-protein and protein-protein interface evolution during CG MD simulations for the dimeric complexes. **A** Lipid-protein interfaces for the three different studied *cis*-dimers. **B** Protein-protein interfaces belonging to the three different *cis*-dimers. The colours in the curves match the outline of the corresponding image in the inset (*black*: head-to-head narrow, *red*: head-to-head wide, *cyan*: back-to-back). The domains in the inset schemes are coloured in the same way as Figure 2.

289 We have tested the robustness of the three *cis*-dimer models through coarse-grained molecular dynamics (MD) simulations (see methods). The final conformations obtained for all three MD simulations stay close to their respective starting models (Supplementary Fig. 1). This observation suggests that the models are relatively stable, and therefore can all be considered as possible molecular assemblies (see detailed analysis in supplementary material). Notably, the dynamics of the closed back-to-back model shows a decrease in the membrane-protein interaction surface as

295 well as an increase in the protein-protein interface (Fig. 3, cyan curve). The latter is a consequence
 296 of the augmented interaction of their transmembrane regions with each other that consequently
 297 decreases the lipid-protein interface. At this point out our study, MD simulations alone do not
 298 allow privileging the validity of any model over the others. For this reason we analyzed the
 299 propensity of each *cis*-dimer to possibly associate with the mitochondrial outer membrane carrier
 300 protein Ugo1 as *cis*-dimerization of Fzo1 has been suggested to involve the participation of Ugo1
 301 (Anton et al., 2011). This 3-membrane spanning factor essential for outer membrane fusion
 302 (Coonrod et al., 2007; Hoppins et al., 2009; Sesaki and Jensen, 2001, 2004; Wong et al., 2003)
 303 interacts with Fzo1 through a region spanning residues 630-703 and 756-843 (Sesaki and Jensen,
 304 2004) and may thus contribute deciphering the differential likelihood of the three *cis*-models.
 305 Interestingly, it is obvious that in the wide and narrow head-to-head dimers, the region required for
 306 potential interactions with Ugo1 is exposed and fully accessible (Fig. 4a, b). In contrast, this region
 307 becomes partially masked and likely less accessible to potential Ugo1 molecules in the back-to-
 308 back model (Fig. 4c). If Ugo1 indeed participates in Fzo1 *cis*-dimers formation, these observations
 309 would thus tend to favour head-to-head models as opposed to the back-to-back configuration.
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 312 **Figure 4. Exposed Ugo1-interacting regions in Fzo1 putative *cis*-dimers.** a, b and c. Final
 313 snapshots from the simulations showing the Ugo1-interacting surfaces (yellow) for each of the *cis*-
 314 dimers. Each Fzo1 monomer is represented with a different colour (magenta or cyan) and the
 315 membrane bilayer is depicted as a brown transparent surface.
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317 **Proposed models for Fzo1 *trans*-tethered oligomers**

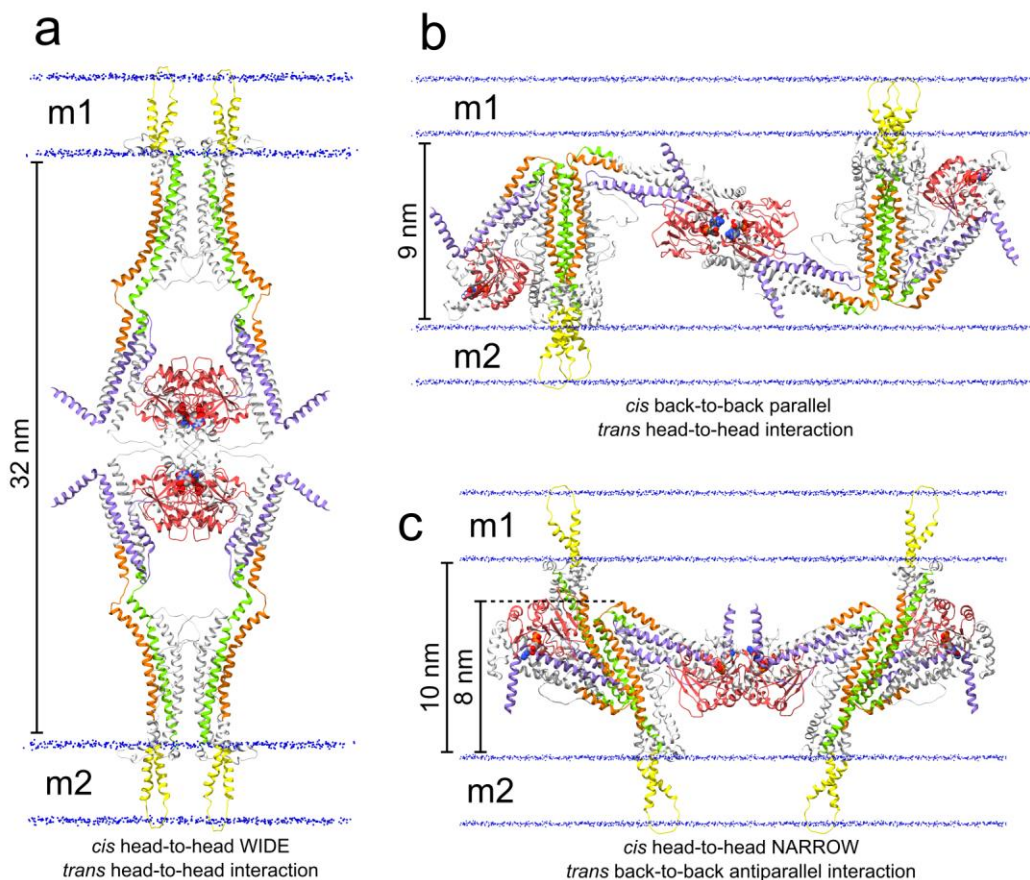
318 Based on the three *cis*-dimers of Fzo1 previously obtained, we aimed at modelling potential
 319 mitofusins *trans*-oligomers. We took here in consideration the possibility that *cis*-dimers from one
 320 membrane could engage in interactions with *cis*-dimers from an opposing membrane through the
 321 availability of either their GTPase or trunk domains. Importantly, the average 8 nm distance
 322 between outer membranes observed during Fzo1-mediated mitochondrial tethering (Brandt et al.,

323 2016) provides an experimental estimation of the membrane separation that Fzo1 *trans*-oligomers
 324 should favour.
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326 Following GTP binding, wide opening of Fzo1 would take place and head-to-head *cis*-
 327 dimerization through GTPase domains would generate competency for *trans*-oligomerization. In
 328 this configuration, *trans* interactions could take place through GTPase domains using an interface of
 329 association distinct from that used during *cis*-dimerization (Fig. 5a). Such a surface of interaction
 330 remains yet to be established experimentally. Moreover, the resulting Fzo1 *trans*-oligomer would
 331 impose a tethering distance of 32 nm between the two juxtaposed outer-membranes, which is four
 332 times higher than the average 8 nm distance experimentally observed (Brandt et al., 2016).

333 In the alternative configuration in which GTP binding would induce narrow opening of
 334 Fzo1, the most exposed surface of *trans*-interaction between head-to-head *cis*-dimers would lie in
 335 the trunk region. Anti-parallel associations reminiscent of those observed in the crystal structure of
 336 the Mfn1 HR2 domain (Koshiba et al., 2004) would take place between dimers from opposing
 337 membranes (Fig. 5c). This would impose a tethering distance of about 10 nm, which is compatible
 338 with the cryo-ET experimental measures (Brandt et al., 2016).

339 In the context of the back-to-back HR-parallel *cis*-dimers (Fig. 2c) GTP binding would
 340 induce the conformational switch of Fzo1, which would allow the GTPase domain to engage in
 341 *trans* association with the GTPase domain of a back-to-back *cis*-dimer from an opposing membrane
 342 (Fig. 5b). Although this Fzo1 *trans*-oligomer imposes a tethering distance of 9 nm which is
 343 compatible with experimental observations, an extensive manipulation of the initial closed
 344 conformation involving hinges 1a and 1b was required to generate the GTPase domain interface.
 345 Without this modification; the starting orientation of the GTPase domain would prevent the
 346 formation of the canonical G-interface observed in the dynamin superfamily (Daumke and Prafke,
 347 2016) and Mfn1 dimers (Cao et al., 2017; Yan et al., 2018), (Supplementary Fig. 2).
 348



349 **Figure 5. Putative model complexes for Fzo1 *trans*-tethering interactions.** a, the Fzo1 models in
 350 open conformations, as suggested from analogy to the BDLP system, interacting through their
 351

352 GTPase domains. **b**, the Fzo1 *trans*-tetramer in which the HRs are oriented in a parallel fashion and
353 the *trans* interaction occurs towards the GTPase domain. **c**, the Fzo1 *trans*-tetramer in closed
354 conformation. Here the interaction occurs towards the GTPase domain as well as through the
355 respective HR domains oriented in an antiparallel fashion. Outer membranes corresponding to two
356 different mitochondria are labelled as m1 and m2. The domains highlighted by color are: *violet*,
357 HRN; *green*, HR1; *orange*, HR2; *red*, GTPase and *yellow*, transmembrane. Phosphorus atoms
358 (*blue*) from lipid bilayer headgroups and GDP nucleotide are depicted in the space-filling
359 representation.

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362 ***Trans*-dimer dynamics.**

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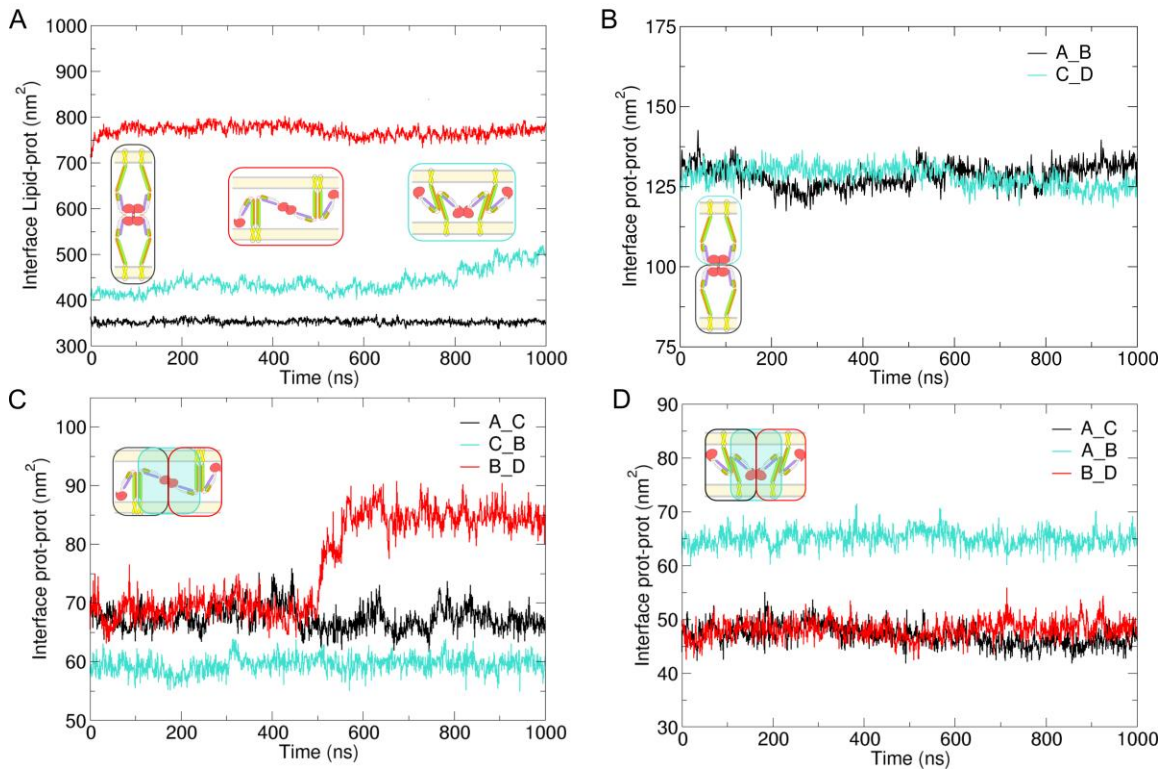
364 The robustness of the tetramers has been tested through coarse-grained molecular dynamics
365 simulations. The final conformations obtained for all three MD simulations show a structural drift
366 with respect to their respective starting models that, although significant (Supplementary Table 2),
367 does not point to major issues in the structure (Fig. 6, 7). Thus, it is possible to infer that the models
368 are stable in a membrane environment, and can therefore be considered plausible (see detailed
369 analysis in supplementary material). When analysing the interface area between protein and lipids,
370 we detected that the head-to-head model with parallel HR interactions has a higher interaction
371 surface with the membrane compared to the other two tetramers (Fig 6a, red line). For this model,
372 we have also observed a jump in the protein-protein interface in one of the two back-to-back *cis*-
373 dimers, as a consequence of an increased interaction between the transmembrane regions of each
374 monomer (Fig 6c, red curve). Interestingly, the head-to-head *trans* antiparallel model showed an
375 increase in membrane curvature during the simulation as a consequence of the strong interactions
376 between the protein terminal regions and lipids as well as a region spanning residues 380-386 in the
377 GTPase domain (Fig. 7c). Even taking in consideration that this model is missing the first 100
378 residues from the yeast mitofusin, the region including residues 380-386 appears to interact strongly
379 in the back-to-back *cis*-dimer as well, where the terminals do not interact with the membrane. An
380 analysis of the distance evolution between opposing membranes for all the tetrameric complexes
381 showed that globally it changed slightly during the MD run without affecting the comparison with
382 experiments. In particular, for the head-to-head *trans* anti-parallel interaction the initial distance
383 between the membranes was 10 nm, that value kept close only in the middle dimer (9.5 nm) after 1
384 μ s, while at the extremes of the dimer the intermembrane distance stabilised at approximately 5.5
385 nm. This narrower value appeared as a consequence of the increased curvature in the membrane
386 caused by the strong protein interactions with the lipid (Fig. 6c, Supplementary Fig. 3).

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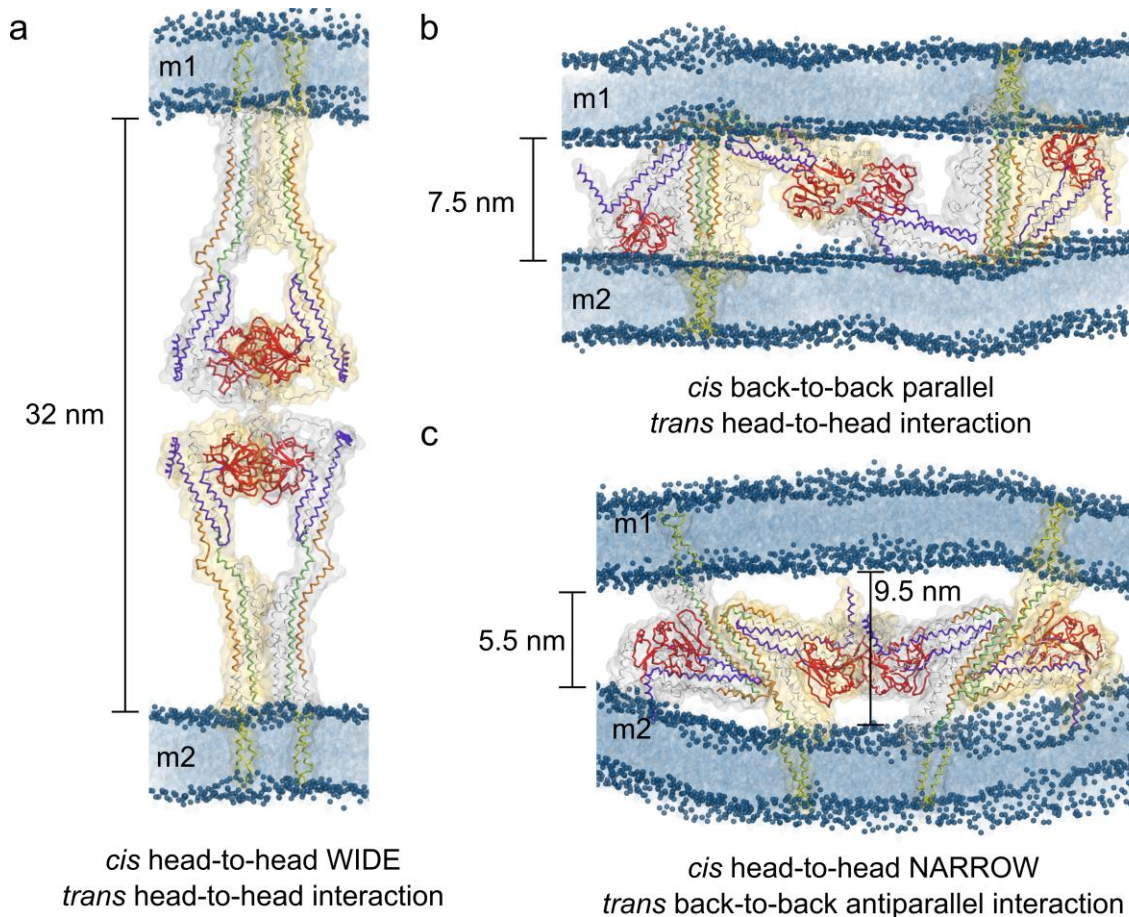
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393 **Figure 6. Lipid-protein and protein-protein interface evolution during CG MD simulations**
 394 **for the tetrameric complexes.** **A** Lipid-protein interfaces for the three different studied tetrameric
 395 complexes (*black*: head-to-head *trans* open, *red*: head-to-head *trans* parallel, *cyan*: back-to-back
 396 *trans* antiparallel). **B**, **C** and **D** depict the evolution of the characteristic monomer-monomer
 397 interfaces belonging to the *trans*-tetramer in open conformation, the *trans*-tetramer in which the
 398 HRs are oriented in parallel and the *trans*-tetramer with HRs oriented in an antiparallel fashion.
 399 Legends A, B, C and D represent each of the monomers in the complex, following the coloring of
 400 image inset.



401
402
403 **Figure 7. Final configurations of the putative model complexes for Fzo1 *trans*-tethering**
404 **interactions after relaxation through molecular dynamics simulations.** a, the Fzo1 models in
405 open conformation, as suggested from the BDLP system analogy, interacting through their GTPase
406 domains. b, the Fzo1 *trans*-tetramer in which the HRs are oriented in a parallel fashion and the
407 *trans* interaction occurs towards the GTPase domain. c, the Fzo1 *trans*-tetramer in closed
408 conformation. Here the interaction occurs towards the GTPase domain as well as through the
409 respective HR domains oriented in an antiparallel fashion. In all figures the domains are colored:
410 violet, HRN; green, HR1; orange, HR2; red, GTPase and yellow, transmembrane. Phosphorus
411 atoms (dark blue) from the lipid headgroups of two mitochondria outer membranes (m1, m2) are
412 depicted as spheres, whereas the tails are highlighted as a blue shadow. Intermembrane distances
413 between the outer phosphate layer of the membranes are indicated.

414 CONCLUSION

415
416 In analogy to the macromolecular spirals formed during DRP-mediated membrane fission
417 (Jimah and Hinshaw, 2019), DRP-mediated membrane fusion may require the formation of macro-
418 oligomers as observed with the Fzo1-dependent docking ring structure (Brandt et al., 2016). Such
419 assemblies require building blocks as previously shown for fission DRPs that associate with their
420 cofactors in the cytosol before recruitment to outer membranes and formation of the spirals (Koirala
421 et al., 2013; Lackner et al., 2009; Mears et al., 2011). In the context of mitofusins, these building
422 blocks would form on the same membrane imposing the assembly of *cis*-oligomers.

423 This rational led us to build 3 *cis*-assembly Fzo1 models that gave rise to 3 distinct *trans*-
424 oligomeric models of mitofusin. Each model involves two main components of mitofusin auto-
425 oligomerization: the GTPase domain interface (Cao et al., 2017; Yan et al., 2018) and the trunk
426 domain interface (Koshiba et al., 2004). A plethora of experimental data demonstrates that the
427 integrity of both regions is indeed essential for mitofusin-mediated mitochondrial fusion (Cohen et
428 al., 2011; Eura et al., 2003; Griffin and Chan, 2006; Hermann et al., 1998; Honda et al., 2005;
429 Koshiba et al., 2004; Rojo et al., 2002; Santel and Fuller, 2001). If considering BDLP, the GTPase

430 domain interface should trigger oligomerisation in *cis* of Fzo1 (Low et al., 2009). Nonetheless, this
431 interface is currently thought to trigger *trans*-oligomerization of mitofusins (Cao et al., 2017; Yan et
432 al., 2018).

433 Only one out of our three final *trans* models employs this strategy. In the back-to-back *cis*-
434 dimers (with parallel-oriented HRs), two dimers from opposing membranes would engage in *trans*-
435 interactions through their GTPase domains (Fig. 2c, and 5b). Yet, the generation of this *trans*-
436 tetramer required an extensive rotation of the GTPase domains around the axis of their four helix
437 bundles to reconstitute the canonical G-interface where GTPase domains mirror each other in other
438 members of the dynamin superfamily (Cao et al., 2017; Daumke and Praefcke, 2016). Moreover, a
439 large area of the Ugo1 binding domain in each Fzo1 monomer was hindered by the HR interacting
440 interface required to form the back-to-back *cis*-dimers (Fig. 4c). This model thus accumulates
441 significant limitations upon confrontation to the experimental literature.

442 The two remaining models employ head-to-head *cis*-oligomerization properties. In this
443 configuration, GTP bound Fzo1 would undergo opening that could be narrow to wide. As seen for
444 the stabilization of GTP-bound fission DRPs with their cofactors (Lackner et al., 2009), this
445 conformational switch around the hinges 1a and 1b would be stabilized by concomitant binding of
446 Ugo1 through the available trunk and a neighbouring mitofusin molecule through the activated
447 GTPase domains. In both wide and narrow models the canonical G-interface is maintained. From
448 then, wide or narrow *cis*-oligomers would engage in *trans*-association with Fzo1 *cis*-complexes
449 from the opposing membrane.

450 The BDLP-like wide oligomers would associate in *trans* through a G-domain interface that
451 is distinct from that employed during *cis*-dimerization. To our knowledge, such an interface has
452 never been observed for other members of the dynamin superfamily. Moreover, these Fzo1 *trans*-
453 oligomers would impose a tethering distance between outer membranes of ~ 32 nm, which is
454 significantly larger than the distance observed experimentally between docked mitochondria (~ 8
455 nm, Brandt et al. 2016). Therefore, similar to the back-to-back system, the wide opening head-to-
456 head model is thus difficult to reconcile with the literature.

457 In contrast, the narrow opening head-to-head mechanism employs a *trans*-oligomerization
458 strategy that turns out to fit particularly well with the past and current literature. The trunk region in
459 the *cis*-oligomers would expose a hydrophobic spine from the HR2 that would be available for
460 putative interactions (De Vecchis et al., 2017). These interactions would take place with an HR2
461 belonging to Fzo1 *cis*-oligomers from an opposing membrane in an anti-parallel fashion. This
462 would be consistent with anti-parallel HR2 interactions observed in 2004 for Mfn1 (Koshiba et al.,
463 2004). Moreover, the tethering distance of 5.5 to 9.5 nm imposed by this Fzo1 oligomerization
464 model would agree with the ~ 8 nm separation between outer membranes observed experimentally
465 twelve years later (Brandt et al. 2016).

466 The narrow opening head-to-head *cis*-oligomerization followed by the antiparallel back-to-
467 back *trans*-associations system thus allows speculating on the possible outcome of the fusion
468 complex. Following GTP hydrolysis, hinges 1a and 1b might restore the displacement of the
469 GTPase domain back to the trunk (i.e. a closed state). Similar to the dynamin BSE domain that may
470 transfer energy from the GTPase domain to the trunk during DRP-mediated fission (Chappie et al.,
471 2010; Daumke and Praefcke, 2016), this movement could induce a sliding of interacting HRs with
472 respect to each other. This would contribute to further reducing the distance between outer
473 membranes to 2-3 nm as previously observed (Brandt et al. 2016). This cycle would reiterate
474 around this initial region of minimal contact to reach the docking stage characterized by an
475 extended area of membrane apposition delimited by the ring-shaped mitochondrial docking
476 complex (MDC).

477 It is tempting to propose that this MDC that stands in regions where the distance between outer
478 membranes reaches 6 to 8 nm (Brandt et al. 2016) corresponds to a macromolecular assembly of
479 Fzo1 oligomers reminiscent of those described in the present study. In addition, our models provide
480 enough detail to derive experimentally testable hypotheses for future work, for instance in terms of
481 possible crosslinks and surface-exposed residues. Conversely our models could be improved based

482 on experimental observations. Regardless of these possibilities we hope that our hypotheses will
483 provide food for thoughts before elucidating the structure of full length mitofusins with their known
484 or yet to be described cofactors as well as their precise oligomerization properties.

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610

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615 **Supplementary:**

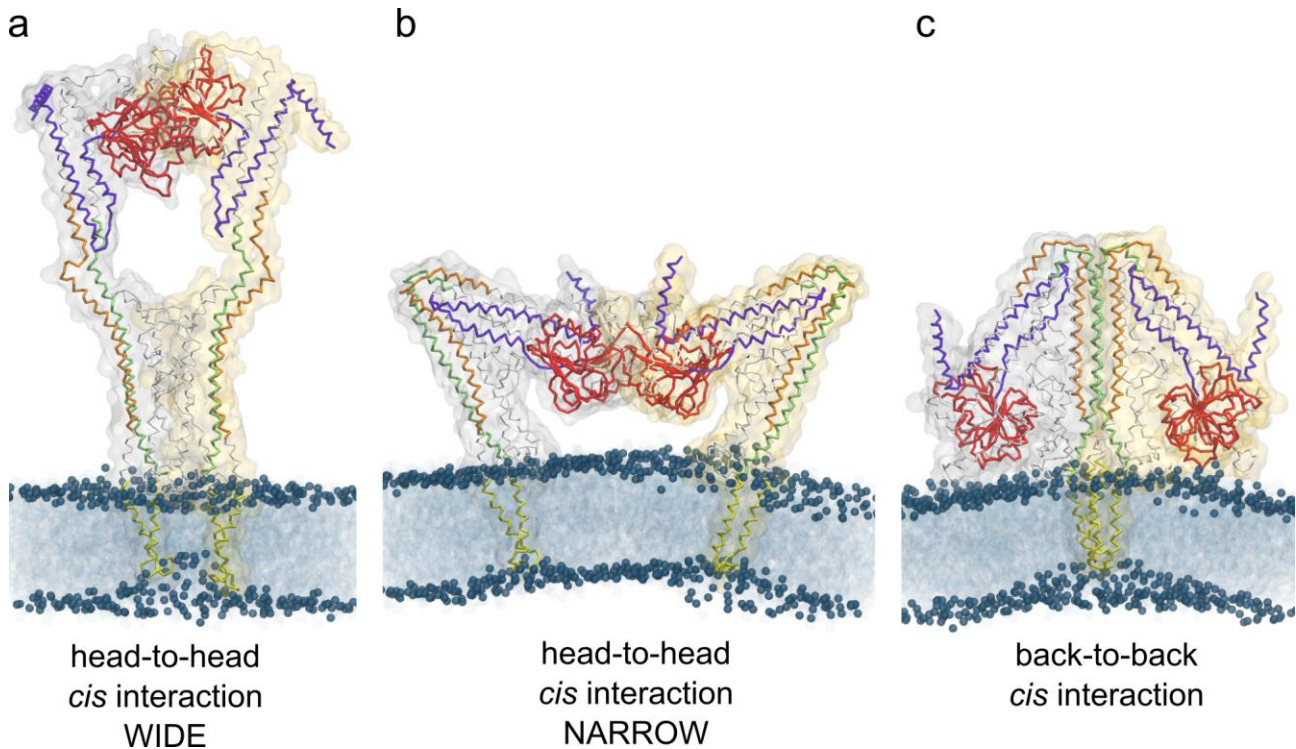
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617 **Supplementary Table 1: Molecular dynamics simulation details for each system**

	Box size (x,y,z [nm])	Number of particles	
		All	Protein + lipid
Dimers			
Head-to-head wide	25.1474 x 25.1474 x 28.9065	157017	27418
Head-to-head narrow	24.6357 x 24.6357 x 29.8118	156752	27058
Back-to-back	25.2349 x 25.2349 x 28.7671	157232	27298
Tetramers			
Head-to-head <i>trans</i>	29.4832 x 29.4832 x 67.4888	523921	78836
Head-to-head <i>trans</i> parallel	49.4896 x 39.5917 x 28.3762	497044	166292
Head-to-head <i>trans</i> antiparallel	30.4625 x 40.6166 x 32.2698	347636	101324

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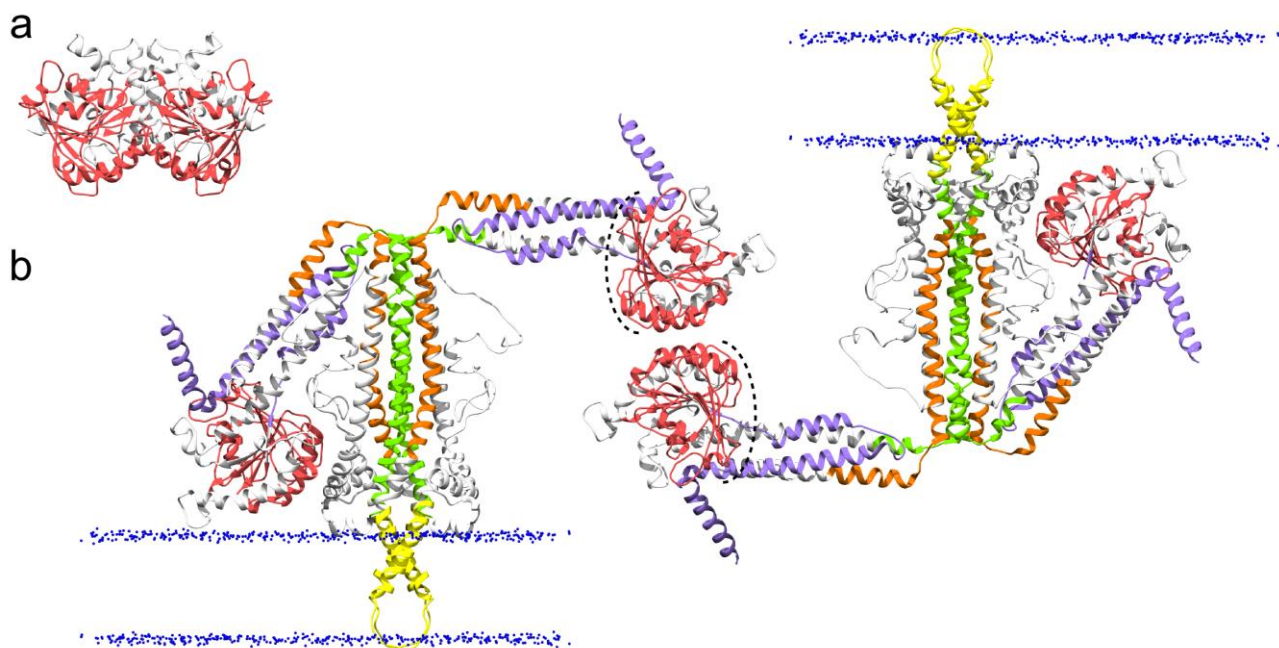
621 **Supplementary Figure 1: Putative membrane-inserted Fzo1 models interacting in *cis***
622 **configuration after molecular dynamics relaxation. a and b.** Wide and narrow head-to-head
623 complexes, respectively. **c.** Back-to-back complex of the closed model. The domains are colored:
624 *violet*, HRN; *green*, HR1; *orange*, HR2; *red*, GTPase and *yellow*, transmembrane. Phosphorus
625 atoms (dark blue) from the lipid bilayer headgroups are depicted as spheres, whereas the bilayer is
626 highlighted as a blue shadow.

627

628 **Supplementary Table 2: RMSD values averaged over the last 100 ns of simulation for each dimer**
629 (Figure 2a, b, c) and tetramers (Figure 3a, b, c). Reported values include a full RMSD calculated
630 over all the backbone particles (BB) as well as by every chain and structural fragment separately.

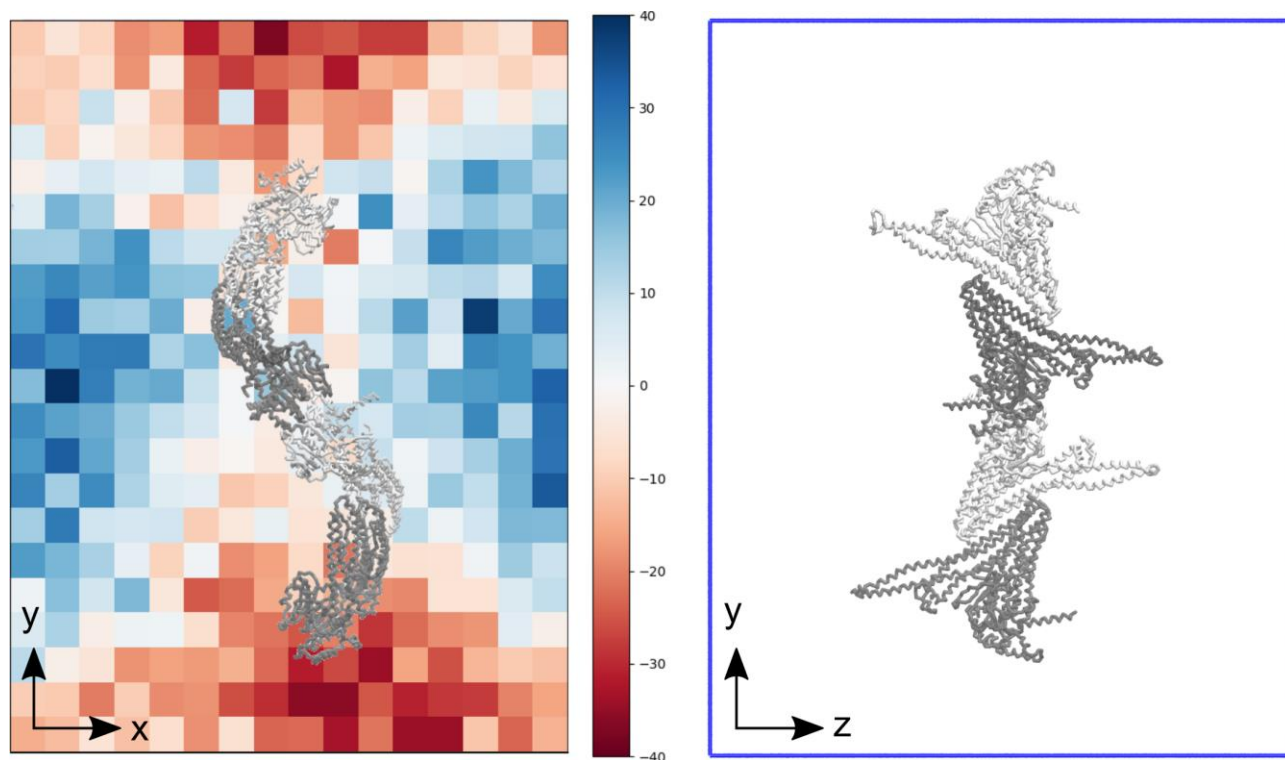
		Figure 2a		Figure 2b		Figure 2c	
		RMSD (nm)	std (nm)	RMSD (nm)	std (nm)	RMSD (nm)	std (nm)
Full protein complex BB RMSD		0.860	0.068	0.401	0.039	0.469	0.013
chain A	all	0.567	0.075	0.332	0.029	0.330	0.016
	frag1	0.261	0.099	0.145	0.036	0.190	0.060
	frag2	0.179	0.013	0.129	0.007	0.233	0.023
	frag3	0.168	0.022	0.137	0.014	0.101	0.012
	frag4	0.431	0.034	0.350	0.021	0.386	0.013
	frag5	0.177	0.040	0.150	0.024	0.132	0.026
chain B	all	0.688	0.062	0.284	0.025	0.463	0.025
	frag1	0.388	0.052	0.290	0.069	0.363	0.094
	frag2	0.169	0.010	0.155	0.011	0.204	0.023
	frag3	0.147	0.023	0.101	0.010	0.109	0.011
	frag4	0.397	0.030	0.266	0.026	0.590	0.030
	frag5	0.243	0.038	0.164	0.024	0.101	0.012
		Figure 3a		Figure 3b		Figure 3c	
		RMSD (nm)	std (nm)	RMSD (nm)	std (nm)	RMSD (nm)	std (nm)
Full protein complex BB RMSD		0.637	0.065	0.303	0.006	0.905	0.068
chain A	all	0.408	0.037	0.220	0.013	0.397	0.035
	frag1	0.236	0.069	0.123	0.010	0.256	0.067
	frag2	0.161	0.011	0.194	0.009	0.185	0.012
	frag3	0.171	0.023	0.095	0.011	0.164	0.017
	frag4	0.335	0.022	0.231	0.028	0.420	0.043
	frag5	0.188	0.033	0.089	0.010	0.153	0.022
chain B	all	0.364	0.030	0.353	0.014	0.283	0.018
	frag1	0.500	0.041	0.351	0.019	0.143	0.037
	frag2	0.197	0.012	0.136	0.006	0.135	0.012
	frag3	0.141	0.023	0.131	0.012	0.097	0.009
	frag4	0.262	0.020	0.390	0.022	0.333	0.021
	frag5	0.266	0.042	0.094	0.013	0.119	0.015
chain C	all	0.513	0.055	0.243	0.011	0.528	0.036
	frag1	0.442	0.112	0.144	0.011	0.249	0.035
	frag2	0.179	0.011	0.155	0.005	0.174	0.015
	frag3	0.180	0.030	0.129	0.014	0.201	0.016
	frag4	0.338	0.021	0.272	0.013	0.421	0.031
	frag5	0.179	0.048	0.162	0.019	0.210	0.021
chain D	all	0.414	0.037	0.245	0.010	0.310	0.029
	frag1	0.361	0.051	0.111	0.009	0.190	0.050
	frag2	0.192	0.010	0.185	0.011	0.193	0.016
	frag3	0.177	0.029	0.104	0.009	0.112	0.011
	frag4	0.362	0.037	0.289	0.014	0.320	0.031
	frag5	0.147	0.027	0.092	0.012	0.131	0.016

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Supplementary Figure 2. **a.** The Fzo1 GTPase dimer construct in which the canonical G-interface commonly observed in the dynamin superfamily (Daumke and Praefke, 2016) is reconstituted. **b.** The back-to-back HR-parallel *trans*-tetramer during the modeling procedure (see Method). The Figure shows how the two GTPase domains that interact in *trans* share an apparent incorrect orientation (compared with **a**). Without an extensive conformational rearrangement that would involve both hinges 1a and 1b, the formation of the canonical G-interface observed in the dynamin superfamily (Daumke and Praefke, 2016) would be impeded. In particular, the dotted line should be located on the same side. The domains are: *violet*, HRN; *green*, HR1; *orange*, HR2; *red*, GTPase and *yellow*, transmembrane. Phosphorus atoms (*blue*) from lipid bilayer headgroups and GDP nucleotide are depicted in the space-filled representation.



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Supplementary Figure 3: Inter-bilayer difference distance matrix (Å). Left panel shows the final difference in distances compared to the initial structure, red regions correspond to regions that

651 got closer in space compared to the initial structure of the production run whereas blue regions
652 represent areas that moved further apart. The right panel shows a rotation of 90 degrees around the
653 y-axis to visualize the position of the complex.
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