A membrane-inserted structural model of the yeast mitofusin Fzo1

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| Family       | Species                | Gene (Id)      | Length | Identity (%) | Similarity (%) |
|--------------|------------------------|----------------|--------|--------------|----------------|
| MFN1 (11481) | **Homo sapiens**       | MFN1 (55669)   | 741    | 19           | 41             |
|              | **Mus musculus**       | Mfn1 (67414)   | 741    | 18           | 43             |
|              | **Gallus gallus**      | MFN1 (424973)  | 740    | 18           | 40             |
|              | **Pan troglodytes**    | MFN1 (460861)  | 741    | 18           | 40             |
|              | **Macaca mulatta**     | MFN1 (709570)  | 741    | 20           | 42             |
|              | **Canis lupus familiaris** | MFN1 (488086) | 742    | 20           | 42             |
|              | **Bos taurus**         | MFN1 (515180)  | 742    | 19           | 42             |
|              | **Rattus norvegicus**  | Mfn1 (192647)  | 741    | 18           | 42             |
|              | **Xenopus tropicalis** | mfn1 (548943)  | 738    | 22           | 43             |
|              | **Danio rerio**        | Mfn1b (393620) | 740    | 21           | 43             |
| MFN2 (8915)  | **Homo sapiens**       | MFN2 (9927)    | 757    | 17           | 41             |
|              | **Danio rerio**        | mfn2 (567448)  | 757    | 18           | 41             |
|              | **Caenorhabditis elegans** | fzo-1 (173990) | 774    | 22           | 40             |
|              | **Drosophila melanogaster** | Marf (31581)  | 810    | 21           | 42             |
|              | **Xenopus tropicalis** | mfn2 (549268)  | 756    | 19           | 42             |
|              | **Pan troglodytes**    | MFN2 (457958)  | 757    | 17           | 41             |
|              | **Macaca mulatta**     | MFN2 (100427191)| 634    | 19           | 43             |
|              | **Canis lupus familiaris** | MFN2 (487439) | 757    | 17           | 41             |
|              | **Bos taurus**         | MFN2 (534574)  | 757    | 17           | 40             |
|              | **Mus musculus**       | Mfn2 (170731)  | 757    | 17           | 41             |
|              | **Rattus norvegicus**  | Mfn2 (64476)   | 757    | 18           | 41             |
|              | **Rattus norvegicus**  | LOC100911485 (100911485) | 757    | 18           | 41             |
|              | **Gallus gallus**      | MFN2 (419484)  | 807    | 18           | 42             |
|              | **Anopheles gambiae**  | AgaP_AGAP001802 (1281313) | 776    | 18           | 40             |
| FZL (95893)  | **Arabidopsis thaliana** | FZL (839566)   | 912    | 23           | 44             |
|              | **Oryza sativa**       | Os05g0390100 (4338678) | 803    | 25           | 45             |
| FZO1 (31469) | **Saccharomyces cerevisiae** | FZO1 (852477) | 855    | 20           | 43             |
|              | **Kluyveromyces lactis** | KLLA0E24179g (2894339) | 825    | 19           | 40             |
|              | **Schizosaccharomyces pombe** | Fzo1 (2539859) | 758    | 20           | 44             |
|              | **Eremothecium gossypii** | AGOS_ABR195C (4619254) | 808    | 20           | 42             |
|              | **Neurospora crassa**  | NCU00436 (3872838) | 918    | 19           | 41             |
|              | **Magnaporthe oryzae** | MGG_05209 (2675586) | 911    | 19           | 40             |
**Supplementary Table 1 | Fzo1 homologue sequences considered in this study.** The source for homologue sequences is the NCBI HomoloGene tool (https://www.ncbi.nlm.nih.gov/homologene, accessed 10-05-2016). The species highlighted by a gray background are those considered in the final target-template alignment. The family name and the HomoloGene identifier are indicated. MFN1, Mitofusin 1, MFN2, Mitofusin 2, FZL, FZO-like and FZO1, fuzzy onions. The identity and similarity with respect to the template BDLP, were obtained by T-Coffee using the *slow_pair* method which is recommended when the sequences are distantly related \(^47\). The BLOSUM45 matrix was used for the similarity score.

| Family Name | HomoloGene Identifier |
|-------------|-----------------------|
| MFN1        |                      |
| MFN2        |                      |
| FZL         |                      |
| FZO1        |                      |
| Mitofusin 1 |                      |
| Mitofusin 2 |                      |
Supplementary Figure 1 | Target-template alignment using the Clustal Omega method considering the whole sequences. The set of 43 sequences from the cyanobacteria (see Methods) were aligned using Clustal Omega\textsuperscript{46}. Subsequently, the generated multiple alignment was merged with the sequence from the target Fzo1 using M-Coffee\textsuperscript{45}.
Supplementary Figure 2 | Target-template alignment using the Clustal Omega method without the first one hundred N-terminal residues from Fzo1. The set of 43 sequences from the cyanobacteria (see Methods) were aligned using Clustal Omega. Subsequently, the generated multiple alignment was merged with the sequence from the target Fzo1 without its first one hundred N-terminal residues, using M-Coffee.
Supplementary Figure 3 | Target-template alignment using T-coffee considering the whole sequences. The set of 43 sequences from the cyanobacteria (see Method) were aligned using T-coffee. Subsequently, the generated multiple alignment was merged with the sequence from the target Fzo1, using M-Coffee.
Supplementary Figure 4 | Target-template alignment using T-coffee without the first one hundred N-terminal residues from Fzo1. The set of 43 sequences from the cyanobacteria (see Methods) were aligned using T-coffee. Subsequently, the generated multiple alignment was merged with the sequence from the target Fzo1 without its first one hundred N-terminal residues, using M-Coffee.
Supplementary Figure 5 | Secondary structure prediction for Fzo1. Continue next page.
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Supplementary Figure 5 | Secondary structure prediction for Fzo1. The name of each predictor used in this study is indicated and the sequence of Fzo1 is aligned to the respective output. The structure submitted to the method DSSP \textsuperscript{77} is the structural Fzo1 model from this study after minimization. The color code is H, α-helix (blue); E, β-sheet (red) and C, coil (yellow). Methods used are: DSSP \textsuperscript{77}, PsiPred \textsuperscript{49}, CONCORD \textsuperscript{48}, PSSpred \textsuperscript{50} and Porter \textsuperscript{51}. 
Supplementary Figure 6 | (a) Predicted disordered region in Fzo1 according the predictor DISOPRED3 [1]. The first disordered region (res 1-86) is located upstream of the N-terminal coiled-coil HRN (res 91-190). The second region (res 415-440) resides between the N- (res 1-415) and C-terminal (res 416-855) halves proposed for Fzo1 [11,13]. The third region (res 663-701) is located in the protein core, whereas a fourth one (res 816-826) is detected in the C-terminal domain, nearby the putative hinge 1b. (b) Root-mean-square fluctuation (RMSF) per residue in all three trajectories (Fzo1.I, cyan; Fzo1.II, green; Fzo1.III, purple). Positions of the putative hinges that encompass the fragments from A to E (TM, transmembrane), are highlighted with asterisks, the names being the same as proposed for the BDLP template [16]. Paler colours and red bars under the plot mark the unstructured/highly flexible regions. Bottom: bar plot indicating conservation per residue of the physico-chemical properties determined by Jalview [85] based on a multiple alignment (Supplementary Fig. 15) of mitofusins belonging to the family of FZO1 (Supplementary Table 1).
Supplementary Figure 7 | Root-mean-square deviation (RMSD) of the protein coordinates as a function of time in MD simulations. Panels (a), (b) and (c) are for the models Fzo1.I, cyan; Fzo1.II, green and Fzo1.III, purple, respectively. In the upper panels, the black line, the red and the one coloured accordingly each trajectory identify values computed considering the whole protein, the highly flexible/unstructured residues (discussed in the text) and the protein core, respectively. The lower panels show the RMSD per-fragment: A (red), B (orange), C (yellow), D (pale green) and E (blue), according to the putative hinges (see also Fig. 1a and Supplementary Table 3). The transmembrane segment is indicated in gray. The values are computed on the alpha carbon atoms with respect to the model after the minimization. In every plot the structure is colored accordingly and represents the last frame from the corresponding simulation time. In the upper panels the structures are depicted in tube representation of varying thickness as a function of the Root Mean-Square Fluctuation (RMSF).
Supplementary Table 2 | Root mean-square deviations.

| Simulation    | Mean (last frame) ± sd |
|---------------|------------------------|
| Fzo1.I        | 4.6 (7.3) ± 2.67       |
| Fzo1.II       | 3.8 (5.7) ± 2.06       |
| Fzo1.III      | 4.6 (6.5) ± 2.68       |

The RMSDs are calculated for the Cα atoms fitting on the same set on the reference structure after the minimization. sd, standard deviation. Values are in Å.
Supplementary Table 3 | Per-fragment root mean-square deviations.

|     | Fzo1.I   | Fzo1.II  | Fzo1.III |
|-----|----------|----------|----------|
| A   | 5.0 (8.0) ± 3.2 | 3.4 (5.1) ± 1.9 | 3.4 (5.3) ± 1.9 |
| B   | 3.6 (5.6) ± 1.9 | 3.4 (5.1) ± 1.8 | 3.1 (4.9) ± 1.6 |
| C   | 1.8 (2.8) ± 0.9 | 1.8 (2.4) ± 0.8 | 1.6 (2.1) ± 0.7 |
| D   | 3.9 (5.9) ± 2.2 | 3.6 (4.8) ± 1.9 | 4.4 (6.4) ± 2.5 |
| E   | 3.5 (5.4) ± 2.0 | 2.2 (2.4) ± 1.0 | 4.9 (8.1) ± 3.2 |
| TM  | 2.2 (3.9) ± 1.1 | 2.6 (3.9) ± 1.4 | 2.4 (4.3) ± 1.3 |

For each protein segment the number of residues is indicated. Statistics are computed on the Cα atoms for each fragment and the values relative to the segment C are calculated without the contribution of the transmembrane domain. The model after the minimization was used as a reference structure. Values are in Å: Mean (last frame) ± standard deviation.
Supplementary Figure 8 | (a) *Ab initio* prediction for the TM helical dimer in Fzo1 using the PREDDIMER server. The models are ordered from the left according to their *F*scor computed by PREDDIMER $^{24}$ 3.113, 3.100 and 2.647, respectively, with associated crossing-angle $\chi$ of 119.7°, 175.1° and -129.7°, respectively. The glycines within the motif GxxxG are in the space-filled representation and residues Lys716 and Ser746 are depicted in stick form. (b) Prediction of the BDLP template orientation with respect to a membrane. The crystal structure PDB-Id 2J68 $^{16}$ has been submitted to the PPM web server $^{67}$. The structure is represented in ribbon mode, the GDP nucleotide in a space-filled representation. The N- (res 2–571) and C-terminal (res 607–695) regions exposed outside of the membrane are in cyan and orange, respectively. The paddle region (res 572–606) is depicted in yellow. The membrane layer is represented by dummy atoms. The predicted embedded residues are 574, 577, 581 and 583, suggesting that BDLP may be a peripheral membrane protein.
Supplementary Table 4 | Residue contacts for the TM domains in the membrane.

| TM1   | TM2            |
|-------|----------------|
|       | Fzo1.I        | Fzo1.II       | Fzo1.III      |
| Leu707|                 |               |               |
| Ile753 (91%) | Leu757 (73%) | Ile753 (56.3%) |
| Thr708|                 |               |               |
| Leu750 (56.8%) | Leu757 (39.8%) | Leu750 (63.3%) |
| Leu709|                 |               |               |
| Leu750 (70.3%) | Ile753 (62.3%) |               |
| Phe711|                 |               |               |
| Ile753 (61.4%) | Ile753 (88.2%) | Ile753 (92.9%) |
| Leu712|                 |               |               |
| Ser746 (36%) | Leu750 (55.2%) | Leu750 (51.8%) |
| Thr715|                 |               |               |
| Gly749 (44.5%) | Ile753 (99.8%) | Gly749 (41.3%) |
| Lys716|                 |               |               |
| Ile742 (55.7%) | Ser746 (68.5%) | Ser746 (73.7%) |
| Gly719|                 |               |               |
| Val741 (67.4%) |               | Ile742 (88%)  |
| Asn720|                 |               |               |
| Ile742 (91%) |               |               |
| Leu723|                 |               |               |
| Val741 (85.7%) | Ile742 (93.2%) | Ile742 (72.8%) |

The Table shows for each monomer in the transmembrane segment which are the corresponding partners between the two TM helices. For each residue position the number of contacts identified are summed and the persistence along each trajectory is indicated. The analysis is conducted without considering the H and the polar atoms N and O. The single common interaction between the replicas is highlighted. TM1 (res 706–726); TM2 (res 737–757).
Supplementary Table 5 | Interactions between protein and membrane.

|        | Fzo.I | Fzo.II | Fzo.III |
|--------|-------|--------|---------|
| POPE   | Ser567| Ser567 |         |
|        | Arg759| Arg759 | Arg759  |
|        | Lys736|        |         |
|        | Leu737|        |         |
|        | Tyr562|        | Tyr562  |
|        | Asp756| Asp756 | Asp756  |
|        | Asp546| Asp546 |         |
|        | Ser738|        |         |
|        | Ser732|        |         |
|        |        |        | Asp563  |
|        |        |        | Ile565  |
|        |        |        | Thr568  |
| POPC   | Gly559| Gly559 | Gly559  |
|        | Lys736|        | Lys736  |
|        | Gly557|        | Gly557  |
|        | Leu558| Leu558 | Leu558  |
|        |        |        | Lys703  |
|        |        |        | Arg759  |
|        |        |        | Lys727  |

Residues interacting through H-bonds over 50% of persistence are indicated. The common interactions between the replicas are highlighted. POPE, palmitoyl-oleoyl-phosphatidylethanolamine; POPC, palmitoyl-oleoyl-phosphatidylcholine.
Supplementary Figure 9 | (a) Cartoon representation of the Fzo1 model and its functional domains. (top) Residue numbers delimiting the domains, (bottom) secondary structure elements are annotated with the Fzo1 mutations performed in this study. The Fig. 4 in main text has been replicated in (a) and extended with data on available mutants across Fzo1 functional domains from the literature ⁹, ¹¹, ¹³, ¹⁵, ³³, ⁸⁶. The point mutations performed in this study are highlighted by larger font size at the very bottom. The color code is cyan, loss of function (LOF), maroon, yeast phenotype is analogous to the wild-type, orange, point mutations that cause a severe LOF only when associated; magenta, residue involved in post-translational modification. Residues considered for the charge swap strategy are connected by a bar. Putative hinge regions are indicated by blue arrows. N- and C-terminal halves are indicated above the secondary structure plot (green and pink, respectively). The topology diagram was generated with the HERA program ⁸³. (b) Level of structuration discussed in this study for the Fzo1 model. The structures refer to the model after the equilibration phase presented also in Fig. 1d. Left, the annotation according to the N-(res 1–415, pink) and C-terminal (res 416–855, green) halves. Right, subdomains across the hinge regions considered in this study.
Supplementary Table 6 | Fzo1 mutants performed in the present study.

| Category            | Residue     | Phenotype              |
|---------------------|-------------|------------------------|
| Deletion fzo1Δ      | fzo1Δ<sup>1−30</sup> | wild-type              |
|                     | fzo1Δ<sup>1−60</sup> | wild-type              |
|                     | fzo1Δ<sup>1−91</sup> | abolish respiration    |
| Point mutation      | K200A       | abolish respiration    |
|                     | K200D       | abolish respiration    |
|                     | K200R       | wild-type              |
|                     | D313K       | abolish respiration    |
|                     | D335K       | abolish respiration    |
|                     | K464D       | abolish respiration    |
|                     | Y490A       | wild-type              |
|                     | Y490K       | wild-type              |
|                     | D523H       | abolish respiration    |
|                     | H780D       | wild-type              |
|                     | E818A       | wild-type              |
|                     | E818P       | abolish respiration    |
|                     | E818R       | wild-type              |
|                     | E819A       | wild-type              |
|                     | E819P       | abolish respiration    |
|                     | E819E       | abolish respiration    |
| Double mutant       | K200D-D313K | abolish respiration    |
| (charge swap)       | D335K-K464D | restore respiration    |
|                     | D523H-H780D | restore respiration    |
Supplementary Figure 10 | Comparison between Fzo1 model and Minimal GTPase Domain (MGD) from Mfn1. (a), (b) and (c) Fzo1 model, Mfn1 crystal fragment (PDB-Id 5GNT) and the partial Fzo1 model based on human Mfn1 (PDB-Id 5GOE), respectively. From left to right MGD domain, homologous salt bridges identified in Fzo1 as well as in the human Mfn1, detail of the GTPase domain showing the homologous residues that may compensate the nucleotide coordination in the G1/P-loop mutant (i.e. K200A and K88A, Fzo1 and Mfn1, respectively) and detail of the GTP binding site indicating the G1–G4 motifs involved in nucleotide stabilization, respectively. The structures were superposed with each other over the correspondent Ca for each fragment using UCSF Chimera. Putative hinge 1a and 1b in Fzo1 are indicated and nucleotides are represented in orange and red stick for yeast and human, respectively. GTPase domain, pink; helices at N-terminal (yellow and orange) and C-terminal (magenta) of the GTPase domain are colored as in Qi et al., 2016 for clarity.
Supplementary Figure 11 | Target-template alignment after the refinement procedure. HRN (N-terminal heptad repeat) violet, res 91-190; GTPase domain red, res 194-373; HR1 (heptad repeat 1) green, res 484-547; transmembrane domain yellow, res 706-757; HR2 (heptad repeat 2) orange, res 769-831. Frames in magenta indicate putative hinges with respect to the BDLp template. In order from the N-terminal: hinge 2a (res 186) hinge 2b (res 435, 441, 443) hinge 1a (res 489-494) and hinge 1b (res 810-815).
Supplementary Table 7 | Analysis of the distances between predicted \( a-d \) positions in Fzo1 heptad repeats.

| \( a,d \) | \( a',d' \) | Fzo1.I     | Fzo1.II    | Fzo1.III   |
|----------|----------|------------|------------|------------|
| M487     | L828     | 12.99 ± 1.69 | 14.27 ± 1.35 | 15.43 ± 0.76 |
| Y490     | S825     | 14.88 ± 1.93 | 16.12 ± 1.68 | 17.72 ± 0.68 |
| E494     | N821     | 20.92 ± 1.92 | 22.64 ± 1.44 | 24.05 ± 1.30 |
| I497     | E818     | 19.82 ± 1.01 | 19.97 ± 1.24 | 21.88 ± 1.29 |
| L501     | I814     | 18.78 ± 1.24 | 17.86 ± 1.72 | 19.79 ± 1.27 |
| L504     | K811     | 21.32 ± 1.22 | 20.45 ± 1.84 | 20.30 ± 1.98 |
| I508     | I807     | 18.18 ± 0.57 | 17.94 ± 0.94 | 18.24 ± 0.84 |
| A511     | S804     | 19.74 ± 0.57 | 19.73 ± 0.73 | 19.91 ± 0.73 |
| C515     | E800     | 19.17 ± 0.81 | 19.44 ± 0.90 | 19.70 ± 1.31 |
| L518     | P797     | 20.28 ± 0.59 | 20.29 ± 0.57 | 20.25 ± 0.72 |
| V522     | V793     | 19.06 ± 0.68 | 19.41 ± 0.47 | 19.19 ± 0.75 |
| M525     | V790     | 19.73 ± 0.85 | 19.59 ± 0.72 | 18.46 ± 1.59 |
| T529     | T786     | 18.73 ± 1.12 | 18.75 ± 0.51 | 17.88 ± 1.14 |
| M532     | A783     | 18.99 ± 1.24 | 19.15 ± 0.66 | 18.30 ± 1.08 |
| N536     | I779     | 18.32 ± 1.73 | 19.15 ± 0.66 | 18.30 ± 1.08 |
| E539     | L776     | 18.06 ± 1.44 | 17.73 ± 0.68 | 17.44 ± 0.56 |
| N543     | K772     | 19.41 ± 1.13 | 19.12 ± 0.87 | 19.01 ± 0.87 |
| D546     | Y769     | 20.08 ± 1.46 | 18.16 ± 0.70 | 19.62 ± 1.43 |

Distances are in Å. The residue at position \( a \) on one chain is packed against the corresponding residue at position \( d \) on the other chain to form \( a-d \) packing. The standard deviation is indicated.
**Supplementary Figure 12 | Heptad repeat domains HR1 and HR2 in the Fzo1 model.** (a) Fzo1 model after the cluster analysis in the Fzo1.I trajectory with the α18 helix labelled. (b) HR1 (green) and HR2 (orange) domains as indicated in (a). The surface represents the side-chains of the predicted a-d positions in the heptads. (c) Helix bundle created by the HR1, HR2 and α18 (white). The latter does not exhibit heptad periodicity and in our model is in close contact with the HR1 towards its a-d positions (green surface). The PCOILS algorithm has been used to predict heptad periodicity (see Methods).
Supplementary Table 8 | Detected salt bridges between the heptad repeat domains in the Fzo1 model.

| HRN/HRN     | HR1/HR1          | HR2/HR2          | HR1/HR2          |
|-------------|------------------|------------------|------------------|
| Glu116 (n)-Arg120 (n+4) | Lys488 (n)-Glu492 (n+4) | Asp792 (n)-Arg795 (n+3) | Arg505-Glu806    |
| Glu116 (n)-Arg130 (n+14) |                        |                  | Glu494-Lys812    |
| Asp128 (n)-Lys132 (n+4) |                        |                  |                  |
| Asp143 (n)-Lys180 (n+37) |                        |                  |                  |

The spacing position for the interhelical interactions is indicated. The persistence is above 60% over the trajectories, the distance cut off is up to 6.5 Å.
Supplementary Table 9 | Target-template homologous residues forming hydrogen bonds with the GDP nucleotide.

| BDLP residue | Donor-acceptor distance (Å) | GDP atom | Donor-acceptor distance (Å) | Fzo1 residue |
|--------------|-----------------------------|----------|-----------------------------|-------------|
| Lys79.N      | 3.04                        | O2B      | 2.99                        | Asn197.N    |
| Arg80.N      | 3.38                        | O2B      | 3.43                        | Thr198.N    |
| Ser83.N      | 2.44                        | O1B      | 2.59                        | Ser201.N    |
| Thr84.N      | 3.14                        | O1A      | 3.15                        | Ala202.N    |
| Thr84.Oγ1    | 3.19                        | O1A      |                             |             |
| Asn238.Nδ2   | 3.21                        | O6       | 3.18                        | Lys370.Nζ   |
| Asn238.Oδ1   | 3.28                        | N7       |                             |             |
| Ala239.N     | 3.25                        | O6       | 3.26                        | Lys371.N    |
| Asp241.Oδ1   | 2.73                        | N2       | 2.70                        | Asp373.Oδ2  |
| Asp241.Oδ2   | 2.81                        | N1       | 2.80                        | Asp373.Oδ1  |
| Ser292.N     | 3.04                        | O6       | 3.04                        | Gly427.N    |
| Ile293.N     | 3.27                        | O6       | 3.21                        | Asp428.N    |

Donor-acceptor distances are calculated with LigPlot+ using a distance cut-off of up to 3.5 Å.
Supplementary Figure 13 | Time series of hydrogen-bond Donor-acceptor distances monitored during the equilibration phase. The colour code is indicated. The dotted lines represent the equilibration steps of varying position restraints on the Fzo1 protein backbone and side-chain atoms (see Methods).
Supplementary Table 10 | Fzo1-human mitofusin 1 homologous residues forming hydrogen bonds with the GDP nucleotide. See next page for caption.

| Fzo1 Model (this study) | 5GNR<sup>18</sup> | 5GNT<sup>18</sup> | 5GOE<sup>19</sup> |
|-------------------------|-----------------|-----------------|-----------------|
| Retained                |                 |                 |                 |
| Asn197N.O2B             | Ser85N.O1B      | Ser85Oγ.O2B     | Ser85N.O2B      |
|                        | Ser85N.O3B      | Ser85Oγ.O3B     | Ser85N.O3B      |
|                        | Ser85Oγ.O3B     | Ser85Oγ.O1A     | Ser85Oγ.O2B     |
|                        | Ser85Oγ.O3A     | Ser85Oγ.O3A     | Ser85Oγ.O3A     |
|                        |                 | Ser85Oγ.O5′     |                 |
| Ser201N.O1B             | Ser89N.O2B      | Ser89N.O1B      | Ser89N.O1B      |
|                        | Ser89Oγ.O2B     | Ser89Oγ.O1B     | Ser89Oγ.O1B     |
|                        | Ser89Oγ.O1A     | Ser89Oγ.O2B     | Ser89Oγ.O2B     |
|                        | Ser89Oγ.O3A     | Ser89Oγ.O2A     | Ser89Oγ.O2A     |
|                        |                 | Ser89Oγ.O3A     |                 |
| Ala202N.O1A             | Ser90N.O2A      | Ser90N.O2A      | Ser90N.O2A      |
|                        | Ser90Oγ.O2A     | Ser90Oγ.O2A     | Ser90Oγ.O2A     |
|                        | Ser90Oγ.O3A     | Ser90Oγ.O3A     | Ser90Oγ.O3A     |
|                        | Ser90Oγ.O2B     | Ser90Oγ.O1B     | Ser90Oγ.O1B     |
|                        | Ser90Oγ.O3A     | Ser90Oγ.O3A     | Ser90Oγ.O3A     |
|                        |                 | Ser90Oγ.O3B     |                 |
| Thr198N.O2B             | Ser86N.O2B      | Ser86N.O2B      | Ser86N.O2B      |
|                        | Ser86Oγ.O2B     | Ser86Oγ.O2B     | Ser86Oγ.O2B     |
|                        | Ser86Oγ.O3A     | Ser86Oγ.O3A     | Ser86Oγ.O3A     |
| Lys370Nζ.O6             | Asn237Nδ2.N7    | Asn237Nδ2.O6    | Asn237Nδ2.O6    |
|                        |                 | Asn237Nδ2.O3A   |                 |
| Average behavior        |                 |                 |                 |
| Lys371N.O6              | Arg238N.O6      | Arg238N.O6      | Arg238N.O6      |
|                        | Arg238Ne.O4′    | Arg238Ne.O4′    | Arg238Ne.O4′    |
|                        | Arg238Ne.O5′    | Arg238Ne.O5′    | Arg238Ne.O5′    |
|                        | Arg238NH2.O5′   | Arg238NH2.O5′   | Arg238NH2.O5′   |
|                        |                 |                 |                 |
| Asp373Oδ1.N              | Asp240Oδ1.N2    | Asp240Oδ2.N2    | Asp240Oδ2.N2    |
| N1                      | Asp240N.N1      | Asp240N.O6      | Asp240N.O6      |
| Asp373Oδ2.N2             | Asp240N.O6      | Asp240N.O6      | Asp240N.O6      |
| Not retained            |                 |                 |                 |
| Gly427N.O6              | Lys286N.O6      | Lys286N.O6      | Lys286N.O6      |
|                        | Lys286N.N7      | Lys286N.O2′     | Lys286N.O2′     |
|                        | Lys286Nζ.O3′    | Lys286Nζ.O3′    | Lys286Nζ.O3′    |
| Asp428N.O6              | Glu287N.O6      | Glu287N.O6      | Glu287N.O6      |
|                        |                 |                 |                 |

<sup>18</sup> Average behavior.
<sup>19</sup> Not retained.
Supplementary Table 10 | Homologous network of hydrogen bond identified in the human mitofusin 1. Homologous residues were identified in the initial target-template alignment (see Methods) as well as after the superposition of the structures indicated. The Fzo1 model represents the centroid of Fzo1.I (see the main text). Residues were subdivided in the three categories discussed in the text. In blue, the analogous interactions, in cyan, interactions retained on the same GDP atom. H-bond were identified using UCSF Chimera 61 (distance cut-off of 3.5 Å and up to 30 degree off-axis angle).
Supplementary Figure 14 | Coordination of the bound magnesium in the GDP binding site. (a) Fzo1 model after the minimization phase, in which the Ser201 (Ser89 in human Mfn1) directly coordinates the cation with a distance of 1.96 Å, as suggested also in the fragment crystal structure from Mfn1 \(^{19}\). Similarly, the homologous Ser41 in dynamin was proposed to act in concert with Lys44 (Lys200 in Fzo1) and Thr65 (Thr221 in Fzo1) in coordinating the bound magnesium, to stabilize the developing charge in the transition state of GTP hydrolysis \(^{37}\). We thus added a magnesium ion in the nucleotide binding site of the Fzo1 model (see Methods in main text), which revealed that Ser201 of Fzo1 possibly plays a role in Mg\(^{2+}\) binding. (b) The structure represents the result of the cluster analysis from the trajectory Fzo1.I. During the simulation time the Ser201 oxydril group was subsequently stabilized over the average of 4.2 ± 0.19 Å between the trajectories. In particular, we observed a change in the coordination from 3 oxygens (1 from α and 2 from β phosphates, see a) to 2 oxygens (1 from α and 1 from β phosphates, see b) with the remaining coordinations being supplied by water molecules. Furthermore, analysis of minimum distances in combination with the number of contacts showed that the water molecules coordinating the Mg\(^{2+}\) at the equilibration phase, remained in place over the simulation time for each replica. Although the placement of the bound magnesium was suggested by available crystal structures (see Method), a template to correctly position the Mg\(^{2+}\) ion for the mitofusin Fzo1 is currently lacking. However, our results suggest a role for this cation in ligand accommodation within the binding pocket as recently suggested for human Mfn1 \(^{18,19}\).
| Protein segment                          | Fzo1 UniprotKB (P38297) | MEMSAT-SVM | OCTOPUS | TMpred |
|-----------------------------------------|-------------------------|------------|---------|--------|
| N-term OUT (exposed to cytosol)         | 1–705                   | 1–703      | 1–734   | 1–706  |
| TM 1                                    | 706–726                 | 704–719    |         | 707–731|
| intermembrane loop (exposed to intermembrane space) | 727–736                 | 720–736    |         | 732–736|
| TM 2                                    | 737–757                 | 737–755    | 735–756 | 737–755|
| C-term OUT (exposed to cytosol)         | 758–855                 | 756–855    | 757–855 | 756–855|

The different predictors used are UniprotKB 41, MEMSAT-SVM 54, OCTOPUS 55 and TMpred 56. TM1 and TM2 are the first and the second transmembrane helix, respectively.
Supplementary Figure 15 | Multiple sequence alignment of Fzo1 homologous identified in this study that belong to the FZO1 subfamily. Continue next page.
Supplementary Figure 15 | Multiple sequence alignment of Fzo1 homologous identified in this study that belong to the FZO1 subfamily. The species considered are *Saccharomyces cerevisiae*, *Kluyveromyces lactis*, *Eremothecium gossypii*, *Schizosaccharomyces pombe*, *Magnaporthe oryzae*, and *Neurospora crassa*. (see also Supplementary Table 1).
Supplementary Table 12 | Characteristics of the simulated system.

| Box size in Å³   | N water | N ions (K⁺/Cl⁻/Mg²⁺) | N lipids (POPE/POPC) | N atoms |
|-----------------|---------|-----------------------|----------------------|---------|
| 102.58 × 102.58 × 164.80 | 36919   | 121/120/1             | 156/156              | 163769  |

Values are the same for all three molecular dynamics simulations Fzo1.I, Fzo1.II and Fzo1.III.
## Supplementary Table 13 | Plasmids used in this study.

| Name (Collection number) | Description | Reference |
|--------------------------|-------------|-----------|
| pRS314                   | CEN, TRP1, Amp | 84        |
| pRS414-FZO1-Myc-FL (MC210) | CEN, FZO1 promoter-fzo1 9MYC, TRP1, Amp | 11        |
| pRS414-1-30 FZO1-Myc-FL (MC380) | CEN, FZO1 promoter-1-30 FZO1-9MYC, TRP1, Amp | This study |
| pRS414-1-60 FZO1-Myc-FL (MC381) | CEN, FZO1 promoter-1-60 FZO1-9MYC, TRP1, Amp | This study |
| pRS414-1-91 FZO1-Myc-FL (MC382) | CEN, FZO1 promoter-1-91 FZO1-9MYC, TRP1, Amp | This study |
| pRS314-FZO1 (MC250)      | CEN, FZO1 promoter-FZO1, TRP1, Amp | 13        |
| pRS314-FZO1 D335K (MC377) | CEN, FZO1 promoter-fzo1 D335K, TRP1, Amp | This study |
| pRS314-FZO1 K464D (MC378) | CEN, FZO1 promoter-fzo1 K464D, TRP1, Amp | This study |
| pRS314-FZO1 D335K-K464D (MC379) | CEN, FZO1 promoter-fzo1 D335K-K464D, TRP1, Amp | This study |
| pRS414-FZO1-13Myc (MC333) | CEN, FZO1 promoter-FZO1-13MYC, TRP1, Amp | 31        |
| pRS314-FZO1 K464D-13Myc (MC442) | CEN, FZO1 promoter-fzo1 K464D-13MYC, TRP1, Amp | This study |
| pRS314-FZO1 D335K-K464D-13Myc (MC444) | CEN, FZO1 promoter-fzo1 D335K-K464D-13MYC, TRP1, Amp | This study |
| pRS314-FZO1 K398R-13Myc (MC334) | CEN, FZO1 promoter-fzo1 K398R-13MYC, TRP1, Amp | This study |
| pRS314-FZO1 D523H (MC400) | CEN, FZO1 promoter-fzo1 D523H, TRP1, Amp | This study |
| pRS314-FZO1 H780D (MC396) | CEN, FZO1 promoter-fzo1 H780D, TRP1, Amp | This study |
| pRS314-FZO1 D523H-H780D (MC397) | CEN, FZO1 promoter-fzo1 D523H-H780D, TRP1, Amp | This study |
| pRS314-FZO1 L819A (MC434) | CEN, FZO1 promoter-fzo1 L819A, TRP1, Amp | This study |
| pRS314-FZO1 L819E (MC433) | CEN, FZO1 promoter-fzo1 L819E, TRP1, Amp | This study |
| pRS314-FZO1 L819P (MC411) | CEN, FZO1 promoter-fzo1 L819P, TRP1, Amp | 13        |
| pRS314-FZO1 E818P (MC406) | CEN, FZO1 promoter-fzo1 E818P, TRP1, Amp | This study |
| pRS314-FZO1 E818R (MC407) | CEN, FZO1 promoter-fzo1 E818R, TRP1, Amp | This study |
| pRS314-FZO1 E818A (MC408) | CEN, FZO1 promoter-fzo1 E818A, TRP1, Amp | This study |
| pRS314-FZO1 Y490A (MC431) | CEN, FZO1 promoter-fzo1 Y490A, TRP1, Amp | This study |
| pRS314-FZO1 Y490K (MC432) | CEN, FZO1 promoter-fzo1 Y490K, TRP1, Amp | This study |
| pRS314-FZO1 Y490K-L819E (MC432) | CEN, FZO1 promoter-fzo1 Y490K-L819E, TRP1, Amp | This study |
| pRS314-FZO1 D313K (MC390) | CEN, FZO1 promoter-fzo1 D313K, TRP1, Amp | This study |
| pRS314-FZO1 K200D (MC391) | CEN, FZO1 promoter-fzo1 K200D, TRP1, Amp | This study |
| pRS314-FZO1 D313K-K200D (MC392) | CEN, FZO1 promoter-fzo1 D313K-K200D, TRP1, Amp | This study |
| pRS314-FZO1 K200A (MC445) | CEN, FZO1 promoter-fzo1 K200A, TRP1, Amp | 13        |
| pRS314-FZO1 K200R (MC427) | CEN, FZO1 promoter-fzo1 K200R, TRP1, Amp | This study |
### Supplementary Table 14 | *Saccharomyces cerevisiae* strains used in this study.

| Name            | Genotype                                      | Reference         |
|-----------------|-----------------------------------------------|-------------------|
| FZO1 (MCY571)   | $MATa\ ura3-1\ trp1-1\ leu2-3,112\ his3-11,15\ can1-100$ $fzo1\Delta::LEU2$ $pRS416\text{-FZO1}$ | This study        |
| FZO1 mdm30 (MCY585) | $MATa\ ura3-1\ trp1-1\ leu2-3,112\ his3-11,15\ can1-100$ $fzo1\Delta::LEU2\ mdm30\Delta::KanMX6$ $pRS416\text{-FZO1}$ | This study        |

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